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MOLECULAR MECHANISMS INVOLVED IN THE MODULATION OF ARYL HYDROCARBON RECEPTOR-REGULATED GENES BY MERCURY, LEAD, AND COPPER

By

HESHAM M. KORASHY

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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ABSTRACT

Co-contamination with complex mixtures of trace metals and environmental carcinogens such as polycyclic aromatic hydrocarbons (PAHs) is a common environmental problem with multiple biological consequences, particularly for metabolizing enzyme systems. Therefore, the objectives of the present study were to; 1) examine the effect of three prominent environmental metal contaminants, mercury (Hg^{2+}), lead (Pb^{2+}), and copper (Cu^{2+}), on aryl hydrocarbon receptor (AhR)-regulated genes, such as cytochrome P450 1a1 (Cyp1a1), NAD(P)H:quinone oxidoreductase 1 (Nqo1), and glutathione transferase a1 (Gsta1), and 2) explore the molecular mechanisms involved. Our results showed that the three metals differentially modulated the expression of AhR-regulated genes at the constitutive and inducible levels. Particularly, we demonstrated that the metals modulate the expression of the *Cyp1a1* gene through an AhR-dependent mechanism at the transcriptional and post-translational levels, in that metals increased *de novo* Cyp1a1 mRNA synthesis whereas significantly decreased the degradation rate of Cyp1a1 protein, respectively. In addition, we showed that the inhibitory effect of metals on Cyp1a1 at the activity level is associated with an increase in heme oxygenase 1 mRNA and a decrease in cellular heme content. On the other hand, the metals induced *Nqo1* and *Gsta1* gene expression through AhR- and nuclear factor erythroid 2-related factor-2 (Nrf2)-dependent mechanisms at transcriptional levels. Also, we provided the first demonstration that redox-transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), are involved in the modulation of AhR-regulated genes by metals. The three metals differentially induced NF-κB and AP-1 DNA binding activities. We demonstrated that activation of NF-κB signaling pathway negatively regulates the expression of AhR-

regulated genes by the metals, whereas activation of AP-1 signaling pathways is required for the metal-mediated effects. In conclusion, our data clearly demonstrate that exposure to metals/AhR ligand mixtures may decrease the carcinogenicity of AhR ligands by decreasing the induction of Cyp1a1 and increasing the induction of Nqo1 and Gsta1 enzymes. These results have a great impact on the understanding of the cellular and molecular mechanisms responsible for the modulation of AhR by metals. This will lead to new strategies to avoid the damage caused by metals and AhR ligands.

This work is dedicated to my parents,

Mohamed Korashy

&

Nariman El-Balboshy

Thank you

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LIST OF ABBREVIATIONS

3MC	3-Methylcholanthrese
7ER	7-Ethoxyresorufin
Act-D	Actinomycin D
AHH	Aryl hydrocarbon hydoxylase
AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
AIP	AhR inhibitory protein
ALDH-3 or Aldh-3	Aldehyde dehydrogenase 3
AP-1	Activator protein 1
ARNT	AhR nuclear translocator
ASTDR	Agency for Toxic Substances and Diseases Registry
BA	Benz[a]anthracene
bHLH	Basic-helix-loop-helix
βNF	β-Naphthoflavone
BSO	L-buthionine-[S,R]-sulfoximine
C/EBPb	CCAAT/enhancer-binding protein b
C12	Hepa 1c1c7 deficient cells
c-AMP	Cyclic adenosine monophosphate
CAR	Androstane receptor
CDNB	1-Chloro-2,4-dinitrobenzene
CEPA	Canadian Environmental Protection Act Registry
CHP	Cumene hydroperoxide
CHX	Cycloheximide
Cu^{2+}	Copper
CYP or Cyp	Cyptochrome P450
CYP1A1 or Cyp1a1	Cytochrome P4501A1
CYP1A2 or Cyp1a2	Cytochrome P4501A2
CYP1B1 or Cyp1b1	Cytochrome P4501B1
CYP2S1 or Cyp2s1	Cytochrome P4502S1

DCF	Dichlorofluorescein
DCF-DA	2′,7′-Dichlorofluorescein diacetate
DCPIP	2,6-Dichlorophenolindophenol
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular-signal regulated kinase
EROD	7-Ethoxyresorufin *O*-deethylation
Gapdh	Glyceraldehydes-3-phosphate dehydrogenase
GSH	Glutathione
GSTA1 or Gsta1	Glutathione transferase A1
H_2O_2	Hydrogen peroxide
HAH	Halogenated aromatic hydrocarbon
Hepa 1c1c7	Murine hepatoma Hepa 1c1c7
HO-1	Heme oxygenase 1
HSP90	90 kDa heat-shock proteins
IL-4	Interleukin-4
JNK	c-Jun NH_2-terminal kinase
Keap1	Kelch-like ECH associating protein 1
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-leucinal
MRE	Metal responsive element
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	Nuclear export signals
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signals
NQO1 or Nqo1	NAD(P)H:quinone oxidoreductase 1
NRE	Negative regulatory elements

Nrf2	Nuclear factor erythroid 2-related factor-2
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons
PAS	Per-ARNT-Sim
Pb^{2+}	Lead
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulphonylfluoride
PPARα	Peroxisome proliferator-activated receptor α
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptor
SRC-1	Steroid receptor coactivator-1
$t_{1/2}$	Half-life
*t*BHQ	*tert*-Butyl hydroquinone
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TEMED	*N,N,N',N'*-Tetramethylethylenediamine
TNF-α	Tumor necrosis factor-α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA responsive elements
UGT1A6 or Ugt1a6	Uridine diphosphate glucuronosyltransferases 1A6
WT	Wild-type
XRE	Xenobiotic responsive element
ε	Extinction coefficient

CHAPTER 1 - INTRODUCTION

1.1 ARYL HYDROCARBON RECEPTOR

1.1.1. Historical Background

Early studies in the 1970s on the regulation of cytochrome P450 (CYP) demonstrated the first observations of stimulation of a mixed function oxygenase enzyme activity in response to benzo[a]pyrene (BaP), an environmental toxicant (Nebert and Bausserman, 1970). This enzyme was then called BaP hydroxylase enzyme (Nebert and Gielen, 1972). Later, the nomenclature aryl hydrocarbon hydoxylase (AHH) was preferred, since the enzyme from cell cultures or from mammalian liver microsomes was able to convert a variety of polycyclic aromatic hydrocarbons (PAHs) to phenolic derivatives and is not specific for BaP (Nebert and Bausserman, 1970). A chronological study showed that benz[a]anthracene (BA), a PAH, was able to bind to cellular materials in the first two minutes of exposure of mouse fetal cells (Nebert and Gielen, 1972). Later, the observations of a variation in the extent of AHH activity in different mice strains suggested the possibility that these different cell types possess a different number of receptor sites for the inducer (Nebert and Bausserman, 1970).

Poland and coworkers in 1976 first identified the presence of a small pool of high affinity stereospecific binding sites (receptors) in the cytosolic fraction from livers of C57BL/6 mice (Poland et al., 1976). This receptor was found to be reversibly bound to radiolabeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated aromatic hydrocarbon (HAH) and the most potent inducer of AHH enzyme activity ever known (Poland et al., 1976). Later studies showed that the TCDD-receptor complex is a large molecule with a stokes radius of 6.6 nm, as determined by gel filtration on calibrated columns, that

sediments at 5.0 S on glycerol gradients (Poland and Kende, 1976; Carlstedt-Duke et al., 1981). Since that time, this receptor has been known as the aryl hydrocarbon receptor (AhR).

1.1.2. Molecular Characterization of the AhR

AhR is a cytosolic ligand-activated transcriptional factor that belongs to basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription proteins that are involved in regulation of cell differentiation and proliferation (Whitelaw et al., 1993; Kerzee and Ramos, 2001). Members of this family also include AhR nuclear translocator (ARNT) protein, the *Drosophila* neurogenic protein single-minded (Sim) and the *Drosophila* circadian rhythm protein period (Per) (Schmidt and Bradfield, 1996; Schmidt et al., 1996). These proteins are characterized by the presence of Per-ARNT-Sim (PAS) domain, a homology region of approximately 250 amino acids (Fig. 1.1).

The *AhR* gene is composed of 11 exons of approximately 30 kB of DNA, where the sequences encoding the HLH domain are contained within exon 2; the PAS domain is encoded by exons 3-9, whereas the binding domain is encoded by exons 7 and 8 (Schmidt and Bradfield, 1996; Schmidt et al., 1996). Sequence analysis studies on the murine *AhR* gene revealed the presence of DNA recognition sites for several transcription factors, such as the xenobiotic responsive element (XRE), metal responsive element (MRE), glucocorticoid responsive element, and activator protein 1 (AP-1) binding sites (Garrison and Denison, 2000), suggesting that these regulatory elements may affect *AhR* gene expression.

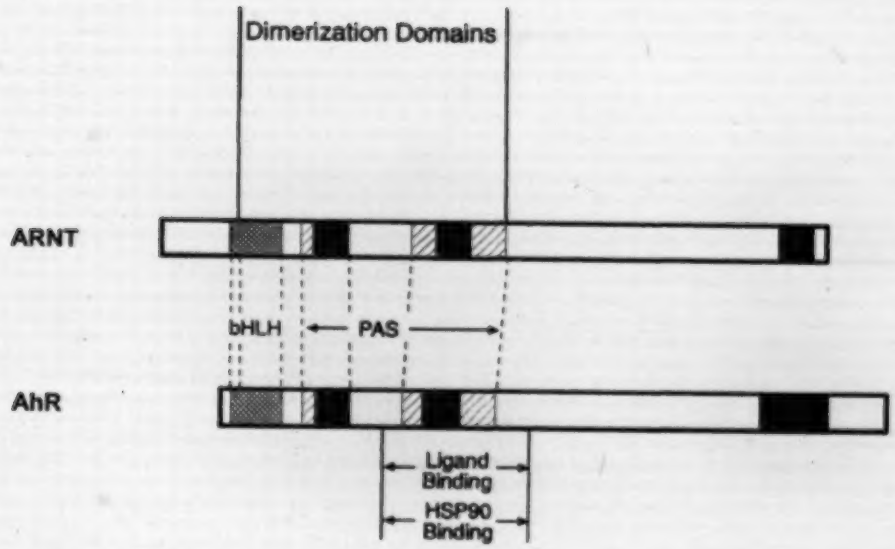

Figure 1.1. Functional and structural domains of mouse AhR and ARNT (Hankinson, 1995).

The physiological effects of bHLH proteins were found to be mediated through the formation of homo- or heterodimeric complexes with other transcription factors for DNA and protein binding activity (Swanson and Bradfield, 1993; Hahn, 1998). Fig. 1.1 shows a schematic representation of the functional domains of AhR and ARNT. The PAS domain in the *AhR* is essential for activation of the AhR and heterodimerization with ARNT. In addition, it has been shown that *AhR* contains nuclear localization and export signals (NLS and NES) in the NH_2-terminal region that play roles in AhR shuttling between the cytoplasm and the nucleus (Ikuta et al., 1998). Generally, the N-terminal half of AhR, which is composed of bHLH and PAS domains, is responsible for ligand binding, dimerization and DNA binding, whereas the C-terminal half is responsible for transactivation (Hankinson, 1995).

The NLS within the AhR is composed of two basic amino acid segments, AhR (13–16:RKRR) and AhR (37–39:KRH), and two protein kinase C (PKC) sites of Ser-12 and Ser-36 are located one amino acid upstream from each of the two segments (Ikuta et al., 2004). It has been demonstrated that phosphorylated NLS abrogates the ligand-dependent nuclear import, while the dephosphorylated NLS promotes its interaction with NLS receptors (Ikuta et al., 2004).

Functional studies in different species showed that the AhR varies in molecular size and amino acid sequence identity. For example, cloning studies showed that the human AhR molecular size is approximately 106 kDa, which is 10 kDa different from that of mouse AhR (95 kDa) (Poland et al., 1987). However, the N-terminus of the human AhR shows a

100% amino acid sequence identity with the murine AhR in the basic region, whereas the C-terminus showed only a 60% identity (Schmidt and Bradfield, 1996). On the other hand, the amino acid sequence identity of ARNT protein is more conserved between species than the AhR (Schmidt and Bradfield, 1996).

Differences in the induction capacity of AhR-dependent genes in response to various PAHs and HAHs have been demonstrated between responsive C67BL/6 and non-responsive DBA/2 mouse strains (Nebert, 1989). Cloning and sequence analysis studies of the AhR from these strains revealed up to 78 amino acid differences at their C-terminus (Poland et al., 1994). Molecular analysis of the coding region of the AhR cDNA from rats sensitive Long-Evans (LE) and resistant Han-Wistar (HW) rats to TCDD toxicity revealed changes in the molecular size, 106 and 98 kDa, respectively, and a change in an amino acid (VAL497 to ALA497) in the transactivation domain (Pohjanvirta and Tuomisto, 1994; Tuomisto et al., 1999). In addition, mutation in exon 10, which modulates ligand-binding properties, has been suggested as a mechanism (Pohjanvirta et al., 1998).

1.1.3. Tissue and Cellular Expression of the AhR

The AhR protein content is known to vary across tissues and cell types at various developmental stages (Swanson and Bradfield, 1993; Hahn, 1998). Generally, AhR protein is expressed in most tissues, with the highest mRNA and protein levels found in the lung, liver, kidney, and placenta, whereas lower levels are expressed in the heart (Carver et al., 1994; Takahashi et al., 1996; Walker et al., 1997; Hahn, 1998; Garrison

and Denison, 2000; Mehrabi et al., 2002). Although AhR and ARNT are expressed in a largely coordinated manner across the tissues, low expression levels of ARNT protein in particular tissues could decrease the sensitivity of that organ to AhR ligands (Carver et al., 1994).

1.2. AhR-REGULATED GENES

To date, the AhR-regulated genes code for four phase I xenobiotic metabolizing enzymes; cytochrome P4501A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1, and four phase II xenobiotic metabolizing enzymes, namely NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione transferase A1 (GSTA1), uridine diphosphate glucuronosyltransferase 1A6 (UGT1A6), and aldehyde dehydrogenase 3 (ALDH3). Although the induction of phase I enzymes bioactivates procarcinogens into their ultimate carcinogenic and genotoxic metabolites, the induction of phase II serves as a detoxification mechanism.

1.2.1. The Phase I AhR-Regulated Genes

The CYPs are single polypeptide membrane-bound hemeproteins that play an essential role in the oxidative metabolism of a great variety of xenobiotic and endogenous compounds (Nebert and Russell, 2002). Microsomal CYPs are attached to the membrane through a hydrophobic transmembrane helix at the N-terminus of the protein (Aguiar et al., 2005). CYPs contain approximately 500 amino acids, of 50 kDa molecular size, and a single heme group coordinated to a cysteine molecule that is essential for thiol-ligand

binding for the heme iron (Poulos, 2005). CYPs are expressed in almost every tissue of the human and in animal hepatic and extrahepatic organs.

CYPs are generally classified based on the primary amino acid sequences of the purified CYP enzyme (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). Members in a gene family generally should share more than 40% amino acid sequence identity. Mammalian members of the same subfamily have a greater than 55% amino acid sequence identity and lie within the same cluster on a chromosome. Therefore, the family is designated by an Arabic number, whereas the subfamily is designated by a capital letter, followed by an Arabic number which represents each individual member (Nelson, 2006; Sim and Ingelman-Sundberg, 2006). In addition, italicized font is usually used to refer to the gene associated with the enzyme (for example, *CYP1A1).* However, italicized small letters are used to describe mouse enzymes (for example, Cyp1a1).

In general, although all different families of CYPs participate in the oxidative metabolism of endogenous molecules such as steroids, fatty acids, and eicosanoids, only the mammalian CYP1, 2, and 3 families are known to be involved in the metabolism of xenobiotics such as pharmacological drugs and environmental contaminants and carcinogens, through different signaling pathways (Ramana and Kohli, 1998). Several genes of these families are highly inducible, particularly by xenobiotics that bind to and activate specific intracellular receptors leading to the initiation of their gene transcription (Barouki and Morel, 2001). Generally, transcriptional activation of most CYPs occurs through at least three nuclear receptor mechanisms: the AhR for CYP1A1, CYP1A2,

CYP1B1, and CYP2S1, the constitutive androstane receptor (CAR) for the CYP2 family; the pregnane X receptor (PXR) for the CYP3 family; and the peroxisome proliferator-activated receptor α (PPARα) for the CYP4 family (Ramana and Kohli, 1998).

1.2.1.1. CYP1A1

Among AhR-regulated CYPs, CYP1A1 is an enzyme which is highly inducible by a broad range of xenobiotics such as PAHs and HAHs via an AhR-XRE-mediated gene transcription pathway (Denison and Nagy, 2003). CYP1A1 is the CYP most capable of bioactivating the toxic and environmental contaminants PAHs and HAHs to carcinogenic metabolites. In fact, a well established link between induction of CYP1A1 and cancer has been reported (McLemore et al., 1990). Several studies have demonstrated that activation of the AhR is the first step in a series of molecular events leading to the induction of CYP1A1. Therefore, the expression level of CYP1A1 is considered to be a useful biomarker of exposure to environmental PAHs ands HAHs (Williams et al., 2000).

Although CYP1A1 is expressed at low levels in extrahepatic tissues, such as lung, and placenta (Shimada et al., 2003a; Shimada et al., 2003b). it is highly inducible in the liver and extrahepatic tissues of most mammalian species, i.e. human, rat, mouse, and rabbit (Guengerich et al., 1982). Examining of the flanking region of the CYP1A gene revealed the presence of a number of sequences that either positively or negatively control the expression of CYP1A. These sequences include c-AMP responsive element, XRE, MRE, AP-1 responsive element, and negative regulatory elements (NRE) (Williams et al., 2000;

Lewis et al., 2006). These results indicate that several factors differentially modulate the expression of the *CYP1A1* gene.

1.2.1.2. CYP1A2

CYP1A2 catalyzes the *O*-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin (Liu et al., 2001; Uchida et al., 2002) and the metabolism of several common compounds such as caffeine, acetaminophen (Tonge et al., 1998), and theophylline (Obase et al., 2003). Furthermore, the constitutive expression of Cyp1a2 in mouse liver but not in AhR knockout mice strongly suggests that CYP1A2 is under AhR regulation (Quattrochi et al., 1998; Uchida et al., 2002). This was supported by the observations that TCDD markedly induced CYP1A2 in primary human hepatocytes through AhR-dependent mechanisms (Zhang et al., 2006). However, an AhR-independent regulation of CYP1A2 has been reported previously (Quattrochi et al., 1998), in which a sequence analysis study of the human *CYP1A2* gene revealed the presence of two sequences homologus to the binding site of the AP-1, in addition to the XRE (Quattrochi et al., 1998).

1.2.1.3. CYP1B1

CYP1B1 is a tumor-related form of CYPs which is constitutively expressed in extrahepatic tissues and is markedly overexpressed in a wide variety of primary tumors (McFadyen et al., 2001a). The presence of CYP1B1 in tumor tissues may be of importance in the modulation of these tumors by anti-cancer drugs (McFadyen et al., 2001b; Murray et al., 2001). In this regard, the high expression levels of CYP1B1 in

tumor tissues, with lack of expression in normal tissues, was found to be partially regulated through proteasomal degradation of the enzyme (Bandiera et al., 2005).

CYP1B1 expression has been shown to be controlled by both transcriptional and post-translational mechanisms (Murray et al., 2001). CYP1B1 can metabolize a range of toxic and carcinogenic chemicals and endogenous substances, and thus plays a critical role in the metabolic bioactivation of numerous procarcinogens such as PAHs and HAHs. Several studies demonstrated that the constitutive and inducible expressions of CYP1B1 mRNA do not correlate with the expression of AhR mRNA. In addition, the constitutive Cyp1b1 mRNA and protein were expressed in ARNT-deficient murine hepatoma cells as compared to wild-type (WT) cells (Eltom et al., 1999). These results suggested that other mechanisms possibly contributed to the regulation of CYP1B1, including non-AhR-mediated pathways and/or post-transcriptional mechanisms.

1.2.1.4. CYP2S1

Although TCDD inducibility was thought to be primarily restricted to the CYP1 family members, Rylander and Rivera first identified a novel CYP, CYP2S1, in human (Rylander et al., 2001) and mouse (Rivera et al., 2002), that exhibited a 10-fold induction of its mRNA levels upon exposure to TCDD (Rivera et al., 2002). Amino acid sequences of the human and mouse CYP2S1 revealed a 70% sequence identity (Saarikoski et al., 2005). Further studies showed that the promoter regions of both human and mouse *CYP2S1* contain XRE core sequences, suggesting that AhR and ARNT mediate the

induction of CYP2S1 by TCDD through binding to XRE, in a manner typical of the CYP1 family (Rivera et al., 2002).

The highest expression of CYP2S1 was determined in the lungs, stomach, and intestine, whereas the lowest levels were observed in the liver and heart (Rylander et al., 2001; Rivera et al., 2002). For example, intraperitoneal administration of TCDD to mouse significantly induced Cyp2s1 mRNA levels in the liver and lungs. Furthermore, murine hepatoma Hepa 1c1c7 and human lung A549 cell lines expressed high basal and TCDD-inducible levels CYP2S1 mRNA levels (Rivera et al., 2002; Saarikoski et al., 2005).

1.2.2. Phase II AhR-Regulated Genes

Phase II metabolizing enzymes, such as NQO1, GSTA1, UGT1A6, and ALDH3, catalyze conjugation reactions necessary for xenobiotic metabolism or the further metabolism of phase I enzyme products (Swinney et al., 2006). Thus, these enzymes play an essential role in the detoxification of xenobiotics and carcinogenic metabolites (Lee and Johnson, 2004; Xu et al., 2005). Several studies have demonstrated a complex regulation of these genes, in which the transcriptional activation of these genes are regulated by both XRE and antioxidant responsive element (ARE) (Friling et al., 1990; Chen and Kunsch, 2004; Miao et al., 2005; Xu et al., 2005).

1.2.2.1. NQO1

Quinone oxidoreductase was first identified by Lars Ernster in 1958 in the rat liver cytosol and designated it as DT diaphorase, currently known as NQO1 (Ernster et al.,

1962). NQO1 is a dimeric flavoprotein expressed constitutively in a wide range of mammalian tissues and cell lines. NQO1 catalyzes the two-electron reduction of several environmental contaminants, electrophilic and endogenous compounds (Jaiswal, 2000). Among the three different forms of the NQOs identified to date, NQO1 is the most extensively studied enzyme. Particularly, NQO1 has been shown to play a critical role in protection against free radicals and mutagenicity, and hence is part of a cellular defense mechanism (Vasiliou et al., 2006). Several studies have demonstrated a direct association between the inhibition of NQO1 activities and increased risk of carcinogenesis (Nioi and Hayes, 2004).

Constitutive NQO1 expression is tissue type-specific, in which maximum induction of the NQO1 mRNA was observed in liver and kidneys followed by lung and the heart (Jaiswal, 2000; Joseph et al., 2000). *NQO1* gene expression is inducible by a wide range of xenobiotics [such as PAHs and HAHs (Jaiswal, 2000)], antioxidants [such as *tert*-butyl hydroquinone (*t*BHQ) (Danson et al., 2004; Nioi and Hayes, 2004; Park et al., 2004)], and metals [such as arsenite (Kann et al., 2005)]. To date, analysis of the 5'-flanking region of the *NQO1* gene has demonstrated the existence of several *cis*-acting regulatory elements that mediate the transcriptional activation of *NQO1* gene, such as XRE, ARE, nuclear factor-κB (NF-κB), and AP-1 (Yao and O'Dwyer, 1995; Rushmore and Kong, 2002; Park et al., 2004). Phenolic antioxidants induce *NQO1* gene expression through an ARE-mediated mechanism by the activation of a cytosolic labile transcription factor, nuclear factor erythroid 2-related factor-2 (Nrf2) (Chen and Kunsch, 2004; Jaiswal, 2004; Nioi and Hayes, 2004).

1.2.2.2. GSTA1

GSTs, formally known as glutathione S-transferases, catalyze the conjugation reactions of active electrophilic metabolites and compounds with the endogenous ligand, glutathione (GSH), which in turn is enzymatically degraded into less toxic substances that are easily excreted from the body, thus protecting cells against various chemical stresses and carcinogenesis (Nguyen and Pickett, 1992; Lamb and Franklin, 2002). Based on the amino acid sequence identity, seven classes of cytosolic GSTs have been identified in mammalian species and designated GSTA (Alpha), M (Mu), O (Omega), P (Pi), S (Sigma), T (Theta), and Z (Zeta) (Hayes et al., 2005; McIlwain et al., 2006). GST isoenzymes within a class share more than 40% amino acid sequence identity, whereas those between classes share less than 25% identity. Although all these enzymes catalyze the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) and thus exhibit GSH peroxidase activity toward cumene hydroperoxide (Hayes et al., 1989; Hayes et al., 2005), the regulatory mechanisms controlling their expression are different.

Among the GST enzymes, analysis of the 5' flanking region of the *GSTA1* gene demonstrated the presence of two main regulatory regions that control the basal and inducible expression of *GSTA1*. A sequence identity similar to the XRE core sequence found in the 5' flanking region of the CYP1A1 was reported (Rushmore et al., 1990; Rushmore and Pickett, 1990). The second regulatory elements found in the *GSTA1* showed no homology with the XRE sequence identity. It was known later as ARE, and is important for the basal expression of the GSTA1 (Rushmore et al., 1990; Rushmore and Pickett, 1990). Numerous chemical agents, such as PAHs, HAHs, and phenolic

antioxidants, have been shown to induce GST enzymes and are classified as monofunctional inducers. However, a number of these agents, such as PAHs and HAHs, are also capable of inducing CYP1A, and therefore classified as bifunctional inducers. These observations strongly suggest that two distinct regulatory proteins participate in the transcriptional activation of *GST* genes. Talaly and coworkers have proposed that bifunctional inducers activate the AhR-XRE pathway, resulting in the transcription of both phase I and II genes (Hayes et al., 2005; Giudice and Montella, 2006). In addition, bifunctional inducers could first be metabolized by the induced phase I, thus converting them into a monofunctional inducer of phase II via the Nrf2-ARE pathway (Nioi and Hayes, 2004; Hayes et al., 2005; Giudice and Montella, 2006).

Several pieces of evidence showed that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic and carcinogenic chemicals. Variations of GST expression have been shown to influence susceptibility to human diseases linked to oxidative stress, such as carcinogenesis (Sheweita and Tilmisany, 2003; McIlwain et al., 2006), asthma, and cardiovascular diseases (Korashy and El-Kadi, 2006b). In this regard, meta-analysis studies suggest that a deficiency of GSTT1 and GSTM genotypes increases the risk of carcinogenesis (Katoh et al., 1998; Sheweita and Tilmisany, 2003; Boccia et al., 2006), whereas overexpression of GSTs protect against various chemical carcinogens (Hayes et al., 1989).

1.2.2.3. UGT1A6

UGTs catalyze the glucuronidation and conjugation of a wide range of endogenous substances, such as serotonin, and xenobiotics, such as the drug paracetamol, planar phenols, aryl amines, and phenolic metabolites of BaP. Based on the amino acid sequences identity, two families of UGTs namely UGT1 and UGT2, have been characterized, (Mackenzie et al., 1997). The UGT1 family is composed of one subfamily UGT1A, whereas the UGT2 family has two subfamilies, UGT2A and 2B (Mackenzie et al., 1997; Meech and Mackenzie, 1997). UGT1A6 is mainly expressed in the liver; however low-to-moderate levels of expression have also been demonstrated in extrahepatic tissues, such as intestine, kidney, testis, and brain (Brands et al., 2000; Shelby and Klaassen, 2006). Several factors, such as genetics, tissue-specific, and environmental factors, have been shown to influence the expression of UGT1A6 (Munzel et al., 2003). Induction of *UGT1A6* gene expression has been shown to be mediated through different signaling pathways, such as the AhR-XRE, Nrf2-ARE, PXR, and CAR (Shelby and Klaassen, 2006).

1.2.2.4. ALDH3

ALDH3 is a cytosolic enzyme which belongs to a family of NADP-dependent enzymes that catalyzes the oxidation of toxic endogenous and exogenous aliphatic and aromatic aldehydes to carboxylic acids (Harrington et al., 1987). Tissue distribution studies demonstrated hepatic and extrahepatic expression of ALDH3. ALDH3 is not constitutively expressed in the normal liver, but it is significantly induced in response to PAHs (Takimoto et al., 1992). However, high constitutive expression levels were

reported in the lungs, stomach, urinary tract, skin, and corneal epithelium. Differential tissue expression suggests that distinct mechanisms are involved in the expression of ALDH3.

The induction of ALDH3 transcription in rat hepatoma H4IIE (Takimoto et al., 1992) and Hepa 1c1c7 (Vasiliou et al., 1992) cells in response to PAHs and HAHs is mediated through an AhR-XRE-dependent mechanism. However, constitutive expression of Aldh3 mRNA is elevated in untreated mutant Hepa 1c1c7 cells lacking a functional Cyp1a1 enzyme; normal expression was restored by introduction of a functional murine Cyp1a1 (Vasiliou et al., 1992). These studies suggest that TCDD-mediated induction of ALDH3 is distinct from the CYP1A1/CYP1A2 metabolism-dependent repression of constitutive gene expression (Vasiliou et al., 1992).

1.3. ACTIVATION OF THE AhR

1.3.1 Ligand-Dependent Activation of the AhR

In the bHLH/PAS superfamily, the AhR is the only protein that requires activation by a ligand. In the absence of ligand, AhR exists primarily in the cytoplasm as part of multimeric protein complex of about 280 kDa (Denison et al., 1986), that is composed of, in addition to AhR, two 90 kDa heat-shock proteins (HSP90) and other AhR inhibitory proteins (AIP) of about 46 kDa (Fig. 1.2) (Denison et al., 1986; Sogawa and Fujii-Kuriyama, 1997). *In vitro* studies showed that the binding site for HSP90 within the AhR is overlapping the ligand-binding site (Whitelaw et al., 1993) and masking the AhR-NLS (Fig. 1.2) (Ikuta et al., 1998; Kawajiri and Fujii-Kuriyama, 2007). Therefore it has been

hypothesized that HSP90 functions to keep the AhR in a conformation capable of high-affinity ligand binding and to prevent its nuclear translocation (Ikuta et al., 1998; Kann et al., 2005).

Because the molecular structure of the AhR is not determined yet, quantitative structure-activity relationship studies are commonly used to gain insights into the nature of the ligand-receptor interactions. Theoretically, there are two hypotheses for AhR interaction with its ligands (Mhin et al., 2002). First, electrostatic interaction, in which effective interaction of the ligand with the receptor depends on the molecular electrostatic potential around the ligand (Mhin et al., 2002). For example, it has been demonstrated that all dioxin compounds that were able to activate the AhR share a unique molecular charge distribution pattern, which was dramatically changed with the chlorination pattern (Mhin et al., 2002). The second hypothesis is based on molecular polarizability and the distance between the receptor and the ligand (Mhin et al., 2002). In this regard, it has been shown that the AhR pocket can bind planer ligands with maximum dimensions of 14 Å × 12 Å × 5 Å, that mainly depending on the ligand's electronic and thermodynamic features (Denison and Nagy, 2003).

Binding of the AhR to ligands causes dissociation of HSP90 and AIP from the activated receptor and subsequent translocation to the nucleus. In the nucleus, the activated AhR heterodimerizes with an 87 kDa nuclear transcriptional factor protein, ARNT (Hankinson, 1995). Although ARNT and the AhR of each species are about 20% identical in amino acid sequence, ARNT does not have any ligand binding capacity and

therefore appears to be free from any repressive effect by HSP90 (Whitelaw et al., 1994; Hankinson, 1995). Some evidence suggests that ARNT promotes dissociation of the AhR-HSP90 complex and targets the AhR to its nuclear site of action (Pollenz, 2002).

The AhR-ARNT complex then binds to a specific DNA recognition sequence, GCGTG, within a responsive element known as XRE (Moore et al., 1993). The XRE is located in the promoter region of a number of genes known as the *AhR* gene battery, which includes *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2S1*, *NQO1*, *GSTA1*, *ALDH3*, and *UGT1A6*. The AhR-ARNT-XRE complex is then capable of initiating regulatory control of these genes in a positive or negative manner (Whitlock, 1999; Pollenz, 2002).

On the other hand, an XRE-independent regulation of phase II genes has been demonstrated through the activation of and binding to a DNA recognition sequences located in close proximity to XRE in the promoter region of the phase II genes known as ARE (Vasiliou et al., 1995; Nerland, 2007). ARE was first identified by Rushmore and Pickett in rat *GSTA1* gene that is responsible for the induction of GSTA1 by electrophilic antioxidants and hydrogen peroxide (H_2O_2) (Rushmore and Pickett, 1990). Vasiliou *et al.*, using gel retardation assay, have demonstrated that AhR can bind to both XRE and ARE consensus sequences in the promoter region of phase II genes (Vasiliou et al., 1995). Further studies have revealed the presence of a number of binding sites for reduction/oxidation (redox)-sensitive transcription factors, such as AP-1 and Nrf2 (Itoh et al., 1997).

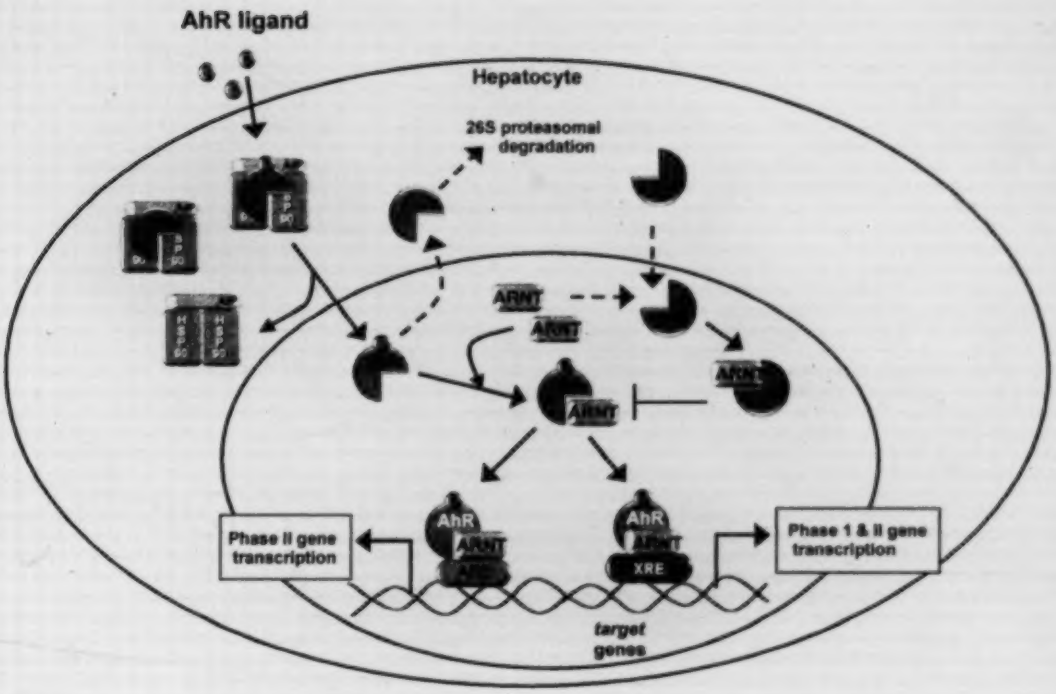

Figure 1.2. Molecular regulation of the AhR and its dependent genes

1.3.2. Ligand-Independent Activation of the AhR

Previous studies showed that inhibition of nuclear export of AhR by leptomycin B, the nuclear export inhibitor, or by mutation of the AhR NES resulted in nuclear accumulation of AhR in the absence of exogenous ligand (Richter et al., 2001). However, binding to ligands increases the rate of nuclear import of AhR but does not eliminate its nuclear export (Richter et al., 2001). These studies suggest that AhR shuttles between the nucleus and the cytosol in the absence of exogenous ligand, and hence activation of AhR could be a ligand-independent process.

An increasing number of reports have demonstrated the ability of several chemical compounds, such as omeprazole, to induce the AhR-dependent gene expression such as that of CYP1A1 without direct binding to the AhR (Backlund and Ingelman-Sundberg, 2004; Lemaire et al., 2004). In this regard, it has been demonstrated that transient expression of AhR and ARNT in AhR-deficient kidney CV-1 cells leads to high levels of AhR-ARNT-dependent luciferase gene expression (Chang and Puga, 1998). Other studies showed that loss of mouse C3H10T1/2 cell–cell contact in the absence of any AhR ligands permits AhR nuclear translocation, activation, and subsequent CYP1B1 induction, whereas the AhR antagonist, α-naphthoflavone, did not affect activation (Cho et al., 2004).

Although the exact mechanisms governing the ligand-independent activation of AhR are still not clear, it has been suggested that metabolic activation of these compounds into AhR ligands or their abilities to stimulate endogenous AhR ligand could play a role

(Sinal and Bend, 1997; Heath-Pagliuso et al., 1998; Schaldach et al., 1999). Moreover, it has been reported that activation of the cyclic adenosine monophosphate (cAMP) mediator (Oesch-Bartlomowicz et al., 2005) or mitogen-activated protein kinases (MAPKs) signaling pathways (Ikuta et al., 2004) increases AhR translocation in a manner somehow similar to, but functionally different from, TCDD-mediated mechanisms. Furthermore, activation of B lymphocytes with CD40 has been shown to activate the AhR with subsequent induction of CYP1A1 in the absence of exogenous ligands (Richter et al., 2001). Another situation in which CYP1A1 can be induced in the absence of ligand is through oxidative stress-mediated effects or induction of cell differentiation that parallels an increase in the AhR transcript (Delescluse et al., 2000). These results suggest the existence of cross-talk between AhR and other signaling pathways that either positively or negatively regulate it.

1.3.3. Classical AhR Ligands

The classical ligands for the AhR include classes of naturally occurring highly toxic and persistent environmental carcinogenic aromatic hydrocarbons, PAHs and HAHs. The most extensively studied members of the PAHs are 3-methylcholanthrene (3MC), β-naphthoflavone (βNF), and BaP, whereas dioxin-related compounds, such as TCDD, represent the prototypes of HAHs (Denison and Nagy, 2003). These classical AhR ligands share structural, functional, and physicochemical features, in that they are planar, aromatic, and hydrophobic in nature (Denison and Nagy, 2003). PAHs and HAHs have been classified as bifunctional, i.e. induce both phase I and II xenobiotic metabolizing enzymes, and monofunctional, i.e. induce phase II only, inducers (Prochaska and Talalay,

1988). In this context, two models have been suggested in that non-metabolizable inducers, such as TCDD, act directly via the AhR to induce both phase I and II enzymes, while metabolizable inducers, such as BaP, act via inducing phase I enzymes specifically to yield metabolites resembling monofunctional inducers (Prochaska and Talalay, 1988). In this regard, it has been demonstrated that 3MC-adduct formation, catalyzed by CYP1A2, mediates the sustained CYP1A1 induction by covalently binding to the AhR (Moorthy et al., 2007).

Binding affinity of ligands to AhR is critically important for their AhR-mediated effects, in which the stronger the binding affinity the greater the toxicity. For example, TCDD is the prototypical ligand and the most potent AhR activator and CYP1A1 inducer ever known. Being resistant to metabolic breakdown, TCDD and other HAHs are more stable and produce sustained effects in the pM to nM range, whereas PAHs are considered metabolically more labile and therefore possess lower AhR affinity and CYP1A1 induction, in the nM to μM range (Denison and Nagy, 2003). In this regard, it has been demonstrated that the toxicity of planar PAHs is extremely sensitive to both the number and position of halogen substituents. For example, TCDD toxicity was drastically decreased on adding non-lateral chlorines or removing lateral chlorines from its structure, suggesting that AhR binding to ligands is sensitive to the chlorination pattern (Mhin et al., 2002)

1.3.4. Non-Classical AhR Ligands

A large number of newly identified AhR ligands whose structures and physiochemical properties significantly differ from those of PAHs and HAHs has been reported (Seidel et al., 2000; Gharavi and El-Kadi, 2005).

The observations that the AhR is activated in the absence of exogenous ligand strongly support the existence of endogenous AhR ligands. For example, it has been shown that hypoxia induces CYP1A1 in rat lungs and liver *in vivo*; this induction coincided with the formation of endogenous AhR ligand. Furthermore, it has been demonstrated that physiological compound(s) contained in serum induce *CYP1A1* gene expression (Guigal et al., 2000; Guigal et al., 2001). Recently, a variety of endogenous compounds has been identified *in vitro* as AhR ligands and activators of the AhR-regulated genes. Those ligands include bilirubin (Sinal and Bend, 1997), lipoxin (Schaldach et al., 1999), and indoles such as tryptophan (Heath-Pagliuso et al., 1998). However, the ability of these compounds to bind to and activate AhR *in vivo* remains to be confirmed. Although the majority of these non-classical AhR ligands are weak CYP1A1 inducers, this list has expanded to include a number of widely prescribed drugs such as omeprazole (Lemaire et al., 2004), primaquine (Werlinder et al., 2001), sulindac (Ciolino et al., 2006), ketoconazole, and itraconazole (Korashy et al., 2007).

1.3.5. Negative Regulation of the AhR and its Regulated Genes

Earlier studies have suggested the existence of NREs and cognate repressor proteins in promoter regions of several AhR-regulated genes, that negatively modulate the

expression of these genes (Boucher et al., 1993; Boucher et al., 1995). Studies on human and rat cells have identified a NRE in the *CYP1A1* gene promoter that appeares to negatively modulate its transcriptional activity by down-regulating a heterologous promoter/enhancer involving specific nuclear protein binding (Jorgensen and Autrup, 1995; Walsh et al., 1996). This was concluded from the fact that mutations in the cognate repressor protein inhibited DNA-protein binding, resulting in a 2- to 3-fold increase in the maximal CYP1A1 inducibility in response to the AhR ligand (Boucher et al., 1995; Piechocki and Hines, 1998).

Moreover, superinducibility of CYP1A1 mRNA by TCDD in Hepa 1c1c7 and human breast cancer MCF10A cells treated with the protein synthesis inhibitor, cycloheximide (CHX), supports the existence and involvement of negative regulatory proteins in the regulation of *CYP1A1* gene expression (Joiakim et al., 2004). In this regard, several studies have identified nuclear transcription proteins, such as OCT-1 and NF-Y (Boucher et al., 1993; Boucher et al., 1995; Sterling and Bresnick, 1996), that constitutively bind to the NREs of the *CYP1A1* gene and hence influence the relative TCDD-induced activity in human hepatoma HepG2, but not human breast cancer MCF7 cells (Jorgensen and Autrup, 1995). Furthermore, the interaction of the AhR-ARNT complex with corepressor transcriptional proteins such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) decreased the XRE binding affinity, as demonstrated by the gel electrophoretic mobility shift assay (EMSA) (Nguyen et al., 1999; Pollenz, 2002)

Moreover, expression of phase II detoxification genes is mediated through a negative regulatory mechanism. Previous studies on rat smooth muscle cells showed that CCAAT/enhancer-binding protein β (C/EBPβ) interacts with ATTGC consensus sequence within the GSTA1 ARE and negatively regulates the *GSTA1* gene expression in response to AhR ligands (Chen and Ramos, 1999; Chen and Ramos, 2000). The repressor effect of C/EBPβ on the inducibility of *GSTA1* was suggested to be a result of C/EBPβ competing with AhR-ARNT for binding to the XRE, or by competing with ARE-Nrf2 for ARE (Chen and Ramos, 1999; Chen and Ramos, 2000). On the other hand, overexpression of a transcription protein, c-Fos, which is known to bind to the binding site of AP-1, repressed the expression of ARE-regulated genes. The negative regulatory effect of c-Fos protein on gene expression was supported by the finding that Nqo1 and Gsta1 protein and activity levels were significantly increased in c-Fos-deficient, but not WT, mice (Wilkinson et al., 1998).

The large intra- and inter-species differences in the susceptibility to TCDD toxicity suggest the possible existence of a negative feedback and protective mechanisms against TCDD toxicity. For example, it has been shown that the LE rat strain is 1000-fold more sensitive to TCDD toxicity than the HW strains (Korkalainen et al., 2004). These observations suggest the existence of a negative cellular factor that is involved in the transcriptional control of AhR-regulated genes, possibly AhR repressor (AhRR) (Fig. 1.2). AhRR, which shares structural similarities with AhR and ARNT, dimerizes with ARNT and thus may compete with the AhR to bind XRE. The resultant AhRR-ARNT complex is capable of binding with XRE, but not to transactivate gene expression

(Swanson and Bradfield, 1993; Mimura et al., 1999; Whitlock, 1999; Korkalainen et al., 2004). Interestingly, real time-polymerase chain reaction (RT-PCR) quantitative analysis of the constitutive expression of AhRR mRNA in LE (Korkalainen et al., 2004) and AhR WT (Bernshausen et al., 2006) mice showed a 3- and 5-fold higher expression in the heart than in the liver, respectively. In addition, it has been suggested that AhRR may facilitate AhR degradation through enhancing the release of the AhR-ARNT heterodimer from the XRE sequence, resulting in repression of AhR function (Gradin et al., 1999).

1.3.6. Physiological and Toxicological Consequences of AhR Activation

Studies on AhR regulation and expression suggest that the AhR possesses xenobiotic-independent functions. Although the AhR has been implicated in several disorders of environmental etiology including atherosclerosis, chloracne, immunosuppression, thymic atrophy, and malignancies (Shimada and Fujii-Kuriyama, 2004; Marlowe and Puga, 2005), it has several physiological functions, as well, such as cell proliferation, apoptosis, and embryogenesis (Marlowe and Puga, 2005).

Early pathological studies using knockout mice have shown that elimination of ARNT expression using a null allele resulted in embryonic death, whereas elimination of AhR expression resulted in pathology of several organs, but not in death (Fernandez-Salguero et al., 1995; Schmidt et al., 1996; Maltepe et al., 1997). This suggests that the AhR-ARNT-mediated signaling pathway plays a vital role in several organ systems. Conflicting data on the role of the AhR in cell cycle progression has been reported in a cell type-dependent manner. For example, it has been shown that AhR inhibited the

growth of MCF7 cells, but promoted HepG2 cell proliferation (Abdelrahim et al., 2003). In addition, it has been demonstrated that the AhR is involved in regulation of normal liver growth (Schmidt et al., 1996) and development of prostate, thymus, and ovaries (Hundeiker et al., 1999), and is necessary for normal developmental closure of the ductus venous (Walisser et al., 2005). In addition, AhR–null female mice showed difficulties in maintaining normal pregnancy (Abbott et al., 1999), suggesting that the AhR has a physiological role in reproductive system. Furthermore, Hushka and coworkers showed that the AhR is involved in the development of the mammary gland; however ligand-dependent activation of the AhR suppresses this process independently of CYP1A1 or CYP1B1 induction (Hushka et al., 1998).

On the other hand, a large-scale mortality analysis study involving 5132 chemical workers routinely exposed to PAHs and HAHs showed a statistically significant trend for the development of cancer and various diseases (Steenland et al., 1999). The AhR has been demonstrated to induce renal disorders, such as hydronephrosis and reduced kidney size, in WT but not in AhR-deficient mice (Falahatpisheh and Ramos, 2003). The role of AhR in carcinogenesis and tumor promotion is clearly established. Acute TCDD toxicity has been shown to act as a potent tumor promoter in a model of liver cancer, and to act as complete carcinogen in chronic toxicity studies (Pitot et al., 1980). BaP has been shown to be a potent carcinogen to experimental animals, in that intrathecal administration of BaP to mice resulted in an increase in the covalent binding to the lung DNA (Mitchell, 1985); however AhR-null mice exposed were resistant to the carcinogenic effect.

One of the proposed mechanisms for the carcinogenic effects of PAHs and HAHs includes increased CYP1-mediated metabolic bioactivation of these compounds or other carcinogens (Schmidt and Bradfield, 1996). These findings were supported by the observations that metabolic activation of BaP to its carcinogenic metabolites was markedly inhibited by antibodies to CYP1A1, but not affected by antibodies against CYP2E1 (Kawajiri et al., 1980). In addition, a proportional correlation has been reported between CYP1 catalytic activity and the activation of BaP to its carcinogenic metabolites (Hietanen et al., 1986). Taken together, the wide range of toxicities following AhR activation suggests a possibility of cross-talk between the AhR and other transcription factors.

1.4. MECHANISMS INVOLVED IN THE MODULATION OF AHR-REGULATED GENES

1.4.1. Transcriptional Mechanisms

Generally, the induction of AhR-regulated genes is mainly controlled at the transcriptional level, through activation of several transcription factors that bind to specific DNA sequences to initiate gene transcription. Inhibition of AhR-regulated gene RNA and protein syntheses using actinomycin D (Act-D) and CHX, respectively, suggest a transcriptional regulatory mechanism is involved in the induction of CYP1A1 (Ramana and Kohli, 1998). In addition, recent studies have demonstrated that the superinduction of the *Cyp1a1* gene by CHX or MG-132, a 26S proteasome inhibitor, is a transcriptional mechanism and reflects a change in the synthesis, rather than stabilization, of Cyp1a1 mRNA (Ma and Baldwin, 2000; Ma and Baldwin, 2002; Joiakim et al., 2004).

On the other hand, ARE-dependent transcriptional activation of *Nqo1* and *Gsta1* genes requires the activation of a CHX-sensitive transcription factor, Nrf2 (Rushmore and Kong, 2002; Xu et al., 2005). In this regard, it has been reported that treatment of Hepa 1c1c7 cells with CHX inhibited the newly synthesized Nqo1 mRNA but did not affect the existing mRNA levels (Eickelmann et al., 1995; Lamb and Franklin, 2002; Ma et al., 2004).

1.4.2. Post-Transcriptional Mechanisms

Steady state mRNA levels reflect a balance between the rate of mRNA synthesis and the rate of mRNA degradation. Therefore, altering the rate of mRNA degradation will definitely influence its steady state concentration and determine how quickly it can be expressed (Lekas et al., 2000). mRNA decay is controlled mainly by the exonucleases that catalyze mRNA, but protected by a specific terminal structure poly(A) tail at the 3' end (Meyer et al., 2004). In addition, other studies have demonstrated that blocking translation has been shown to stabilize mRNA (Meyer et al., 2004). The stability of Mrna of CYPs is usually reflected by their mRNA half-lives ($t_{1/2}$). A previous study on HepG2 cells investigating the decay of CYP1A1, CYP1A2, and CYP1B1 mRNAs after treatment with TCDD showed that CYP1A2 and 1B1 are long-lived CYPs with an approximate $t_{1/2}$ of greater than 24 h (Lekas et al., 2000). In contrast, CYP1A1 mRNA decays quickly, with a $t_{1/2}$ of approximately 2.4 h in HepG2 cells. The rapid decay of CYP1A1 was associated with a rapid loss in poly(A) tail length, suggesting that deadenylation is the first step in the decay pathway (Lekas et al., 2000).

Post-transcriptional regulatory modulation of the AhR-regulated genes is still less well understood. Lee and Safe have demonstrated that inhibition of CYP1A1 mRNA expression in T47D cells in response to resveratrol, a polyphenolic plant extract, is attributed to increased rate of CYP1A1 mRNA degradation (Lee and Safe, 2001). In addition, the adrenal steroid hormone dehydroepiandrosterone decreased CYP1A1 mRNA stability in MCF7 cells by increasing its degradation rate (Ciolino and Yeh, 1999).

1.4.3. Translational and Post-Translational Mechanisms

Post-translational modification was defined as any differences between functional protein and linear polypeptide sequence encoded between the initiation and the termination codons of its structural gene (Han and Martinage, 1992). Most of these amino acid modifications occur after release of polypeptide from the ribosome during the biosynthesis of proteins (Han and Martinage, 1992). Such modifications include noncovalent incorporation of cofactors to form an oligomeric protein and covalent modifications that include cleavage of single peptide and/or altering amino acid residues, such as phosphorylation, glycosylation, and methylation (Aguiar et al., 2005).

1.4.3.1. Phosphorylation

Protein phosphorylation is a reversible cellular process responsible for transfer of phosphate from the adenosine triphosphate (ATP) molecule to the acceptor protein via protein kinases and phosphatases (Han and Martinage, 1992). The most commonly phosphorylated amino acids are serine (Ser), therionine (Thr), and tyrosine (Tyr) (Han

and Martinage, 1992). In the early 1980s, Pyerin and coworkers were the first to demonstrate the possible involvement of phosphorylation in the modulation of CYP genes. The first evidence was the finding that combining purified rabbit CYP2B4 with purified protein kinase A (PKA) phosphorylated Ser128 amino acid (Pyerin et al., 1983; Pyerin et al., 1987). Furthermore, the topological localization of CYP and PKA in the same cellular endomembrane fraction further support the possibility that CYP2B4 will be a substrate of PKA (Oesch-Bartlomowicz and Oesch, 2005).

Furthermore, it has been shown that incubation of hepatocytes isolated from phenobarbital-treated rats, to induce CYP2B1/2, with glucagon, as a stimulant of PKA and c-AMP, resulted in increased incorporation of radiolabeled phosphate in CYP2B1/2 enzymes, as demonstrated by Western blot analysis (Bartlomowicz et al., 1989). However, this was accompanied by a marked decrease in the catalytic activity, while neither a change in CYP2B1 protein levels nor an increase in enzyme inactive P420 forms were observed, suggesting that loss of activity is not mediated through phosphorylation-dependent degradation of protein (Oesch-Bartlomowicz et al., 2001).

Studies on phosphorylation of CYP2E1 showed controversial results. It has been shown that c-AMP-dependent phosphorylation caused a dramatic decrease in both activity and protein degradation; however PKA-dependent phosphorylation of CYP2E1 leads to a marked decrease in the activity without an increase in the rate of protein degradation (Menez et al., 1993).

In contrast to the effect of phosphorylation on CYP2B1/2 or 2E1, the mutagenicity of CYP1A1- and 1A2-formed metabolites in rat liver hepatocytes was significantly reduced by the Ser/Thr protein phosphatase inhibitor, ortho-vanadate (Pyerin et al., 1983; Oesch-Bartlomowicz et al., 2005). However, no incorporation of the radiolabeled ATP pool into CYP1A1 or 1A2 was detected. These observations strongly suggest that the AhR may undergo changes upon PKA activation. This was supported by the observations that treatment of Hepa 1c17 cells with c-AMP resulted in activation of AhR and subsequent translocation to the nucleus (Oesch-Bartlomowicz et al., 2005). In addition, studies on the regulation of CYP1A1 showed that AhR-ARNT heterodimerization requires phosphorylation of only ARNT, whereas binding of the AhR-ARNT to XRE requires phosphorylation of both AhR and ARNT proteins (Pongratz et al., 1991; Berghard et al., 1993).

1.4.3.2. 26S Proteasomal Degradation

The 26S proteasome is a ubiquitous biological multiple protein complex that consists of different subunits. The complex plays a critical role in protein degradation, and hence has a critical function in many cellular processes such as cell cycling, differentiation, apoptosis, and catabolism of abnormal proteins (Han and Martinage, 1992). Nuclear and cytoplasmic proteins are normally labeled with a 76 amino acid polypeptide called ubiquitin (Han and Martinage, 1992). Thereafter, the polyubiquitinated protein will be recognized by the proteasome complex to degrade the target protein into small peptides and cleave ubiquitin to be recycled.

Treatment of Hepa 1c1c7 cells with TCDD shortened the AhR $t_{1/2}$ from 28 h to 3 h (Ma et al., 2000; Fujii-Kuriyama and Mimura, 2005). The down-regulation of AhR in response to its ligands is attributed to the ubiquitination of AhR protein through the 26S proteasome pathway (Eltom et al., 1999). This is supported by the observations that inhibition of the 26S proteasome pathway in Hepa 1c1c7 cells, using MG-132, increases the AhR and ARNT protein levels and hence enhances the induction of Cyp1a1 gene expression (Davarinos and Pollenz, 1999). Furthermore, CYP1A1 luciferase activity in cells treated with TCDD plus MG-132 has shown a 5 to 13-fold induction with a 40% increase in the peaking time, as compared to cells treated with TCDD only (Fujii-Kuriyama and Mimura, 2005).

1.4.3.3. Cellular Heme Content

CYP enzymatic activity levels could be enhanced or suppressed by the cellular heme contents. Several studies have shown that the mechanism of CYP monooxygenase induction is attributed to enhancing of δ-aminolevulinate synthase, a rate-limiting step in the biosynthesis of heme (Lavrovsky et al., 1993). Furthermore, modulation of the expression of HO-1, a rate-limiting step in heme degradation, has been shown to alter cellular heme content and hence the enzyme activity (Kikuchi et al., 2005). Therefore, it seems that the balance between these two pathways could determine the level of CYP enzyme activity.

HOs are stress-responsive enzymes that catalyze the degradation of the porphyrin ring to yield bilivurdin, free heme iron, and carbon monoxide (Kikuchi et al., 2005). Three

different HO isoenzymes, HO 1-3, have been identified to date; they are ubiquitously expressed in a wide range of mammalian tissues (Lavrovsky et al., 1993; Kikuchi et al., 2005). Of those isozymes, HO-1 is the inducible form that anchores to the endoplasmic reticulum membrane via a stretch of hydrophobic residues at the C-terminus (Schuller et al., 1998). Expression of HO-1 is induced by oxidative stress stimuli, such as hypoxia, inflammation, heavy metals, and hydrogen peroxide.

1.4.4. Oxidative Stress

AhR-mediated toxicological effects have been shown to be mediated through the oxidative stress, characterized by an increase in cellular oxidation state to produce an oxidative stress response. Therefore, increased production of ROS and activation of several redox-sensitive transcription factors are considered to directly regulate the expression of AhR-regulated genes.

1.4.4.1 ROS

ROS production has been shown to be one of the mechanisms by which CYP1A1 induction leads to toxicity. It has been shown that during CYP catalytic cycles, monoxygenase enzymes release free radicals, such as H_2O_2. Subsequently, this causes oxidation of several biological macromolecules, such as DNA and proteins. This is supported by the observations that TCDD and BaP cause oxidative stress in various tissues (Barouki and Morel, 2001).

Furthermore, it has been shown that AhR ligands increase ROS production in Hepa 1c1c7 cells through an AhR-dependent mechanism. This was accompanied by a decrease in the Cyp1a1 catalytic activity but not mRNA or protein expressions (Elbekai et al., 2004). The AhR ligand-mediated decrease in Cyp1a1 activity was reversed by the antioxidant *N*-acetylcysteine, suggesting a role for ROS in the modulation of AhR-regulated genes (Elbekai et al., 2004). Mechanistically, Oxidative stress plays a critical role in the CYP1A1 auto-regulatory loop in which generation of ROS as a result of induction of CYP1A1 activity activates the NF-κB signaling pathway that suppresses AhR activation and hence suppression of its gene promoter (Barouki and Morel, 2001).

1.4.4.2. Redox-Sensitive Transcription Factors

With the wide range of toxic responses to PAHs and HAHs from cell proliferation to carcinogenesis, some of which are not directly AhR-dependent, several reports have suggested that the AhR is likely to interact with other transcription factors to cause such diverse effects (Tian et al., 2002). Although some of PAHs elicit unique signal transduction pathways, it has been shown that most of these ligands could trigger other common signaling pathways in the cells. More than 20 redox-sensitive transcription factors have been identified and characterized (Lyakhovich et al., 2006). These transcription factors are responsible for changes in the redox status of the cell in response to stimulants. Among these factors, Nrf2, NF-κB, and AP-1, have been shown to influence expression of several genes that alter the activity of numerous metabolic processes.

1.4.4.2.1. Nrf2

Through an XRE-independent process, recent studies have shown that gene expression of phase II metabolizing enzymes is regulated by a labile protein transcriptional factor, Nrf2. These studies have labeled Nrf2 as the central transcription factor involved in the regulation and expression of many antioxidants and detoxifying phase II enzymes, such as NQO1 and GSTA1, against oxidative damage. In Nrf2-null mice, it has been shown that inducible, but not constitutive, *Nqo1* and *Gstp* gene expressions were abolished, whereas in AhR- and Nrf2-double knockout mice, both constitutive and inducible expressions of *Nqo1* and *Gstp* genes were completely inhibited (Noda et al., 2003; Zhu et al., 2005). These results not only support the notion that AhR- and Nrf2- mediated pathways could play an integral role in the regulation of *Nqo1* and *Gsta1* genes, but also suggest the existence of cross-talk between these pathways. This conclusion was supported by the observations of Ma and Marchand who showed that *Nrf2* gene expression is directly regulated through AhR activation and *NQO1* gene expression is controlled by CYP1A1 activity (Ma et al., 2004; Marchand et al., 2004).

Several pieces of evidence support a direct linkage between AhR and Nrf2. First, *Nrf2* is a target gene for the AhR, in which three functional XRE and two ARE have been identified in the mouse, rat, and human *Nrf2* promoter (Miao et al., 2005; Kohle and Bock, 2007). This is supported by observations that TCDD increased Nrf2 protein levels in a time-dependent manner (Miao et al., 2005), and that studies using Nrf2-deficient cells revealed that induction of NQO1 by TCDD requires functional Nrf2 (Ma et al., 2004). Second, Nrf2 can be activated indirectly by Cyp1a1-generated highly reactive

electrophiles that mediate the induction of phase II enzymes such as NQO1 (Marchand et al., 2004; Miao et al., 2005). Third, a direct interaction between ARE-XRE and Nrf2-ARE signaling pathways has been characterized. Sequence analysis of the enhancer region of mouse *Nqo1* showed that the putative ARE and XRE sequences are located closely to each other, which may suggest a possible functional overlap between their mediated signaling pathways (Ma et al., 2004). Several scenarios have been proposed, in that ARE and XRE function as a composite response element to which both AhR and Nrf2 bind and mediate the induction of NQO1 by TCDD. In addition, AhR and Nrf2 may interact with each other directly or through an adaptor protein; such interactions are required for induction of NQO1 by TCDD (Vasiliou et al., 1995; Ma et al., 2004; Kohle and Bock, 2007).

1.4.4.2.2. NF-κB

NF-κB is a family of transcription factors that plays a critical role in regulating gene expression (Shen et al., 2005). The NF-κB family is composed of six known proteins, NF-κB1, NF-κB2, RelA, RelB, c-Rel, and v-Rel, that can form homodimers as well as heterodimers with each other to bind to enhancer sequences (Tian et al., 1999; Tian et al., 2002). Among these dimmers, NF-κB1/RelA, which refers to an NF-κB complex, is a transcriptionally active complex that was first identified as a transcriptional enhancer of the gene expression of immunoglobulin k light chain in B cells (Sen and Baltimore, 1986), and hence the term NF-κB.

Regulation of NF-κB is a complicated process, in that a wide range of stimuli, such as bacterial lipopolysaccharide (LPS), viruses, oxidative stress, and ultraviolet light trigger NF-κB activation through different mechanisms. For example, although most of these stimuli trigger the NF-κB signaling pathway by activating the IκK pathway, signal cross-talking between NF-κB and other transcription factors or MAPKs has been reported (Wullaert et al., 2006). In this context, several studies have shown that NF-κB participates in many of the physiological as well as toxicological responses mediated by the PAHs and HAHs. Coimmunoprecipitation assays in Hepa 1c1c7 (Tian et al., 1999) and human breast cancer (Kim et al., 2000) cell extracts demonstrated physical and functional interactions between AhR and the RelA subunit of NF-κB. These studies suggested that activation of one signaling pathway could significantly down-regulate the other. This has been demonstrated experimentally in Hepa 1c1c7 cells in which activation of NF-κB suppressed the expression of Cyp1a1 at the transcription level (Ke et al., 2001).

Although it is not clear whether such interaction occurs at the cytoplasmic or nuclear levels, several studies suggested that the interaction of NF-κB and AhR primarily occurs in the cytoplasm since ARNT was not found to be dimerized with RelA in a ligand-dependent manner (Tian et al., 1999). It has been reported that unactivated AhR and NF-κB in the cytoplasm are kept away by being sequestered by their inhibitory proteins, HSP90 and IκB, respectively. However, once activated by TCDD and tumor necrosis factor-α (TNF-α), AhR and NF-κB, respectively, would then interact (Tian et al., 1999). Although the details of cytoplasmic interactions of RelA and AhR are still undetermined, transient transfection of Hepa 1c1c7 cells with AhR did not alter IκB levels, suggesting

that the repressive effects are not mediated through the induction of IκB (Tian et al., 1999). Other studies suggested PKA- and PKC-dependent mechanisms (Zhong et al., 1997; Tian et al., 2002).

On the other hand, the observations of a competition between RelA and AhR for binding to transcriptional coactivators and corepressors strongly suggests a nuclear cross-talk between AhR and NF-κB. Coactivators, such as steroid receptor coactivator-1 (SRC-1) increase histone acetylation of the chromatin, result in transcriptional activation, whereas corepressors, such as SMRT, increase histone deacetylase activity, causing transcriptional repression. Since these coactivators and corepressors operate in a balanced equilibrium, activation of one pathway causes repression of the other pathway. This was recently supported by Tian and coworkers who demonstrated that activation of NF-κB by TNF-α suppressed *Cyp1a1* gene expression through abolishing histone acetylation, which is an initial step for gene expression, resulting in inactivation of the Cyp1a1 promoter (Ke et al., 2001). Furthermore, the suppressive effect of βNF, a potent AhR ligand, on κB-enhancer driven luciferase reporter gene was reversed by the AhR antagonist, α-naphthoflavone (Tian et al., 1999). On the other hand, several studies showed that induction of the *NQO1* gene in human colon adrenocarcinoma HT29 (Yao et al., 1997) and Hepa 1c1c7 (Park et al., 2004) cells by the chemoprotective compounds mitomycin C and baicalin is mediated through the activation of both NF-κB and AP-1 signaling pathways.

Another postulated mechanism for the suppression of AhR by NF-κB activation is through activation of AhRR. In this regard, NF-κB binding site (κB) was found in the promoter region of AhRR; therefore activation of NF-κB will result in induction of AhRR expression that heterodimerizes with ARNT and subsequently suppresses AhR activation and the expression of its regulated genes (Baba et al., 2001).

1.4.4.2.3. AP-1

AP-1 is a transcriptional factor that belongs to a family of basic domain/leucine zipper (bZIP) proteins involved in a wide range of physiological and pathological conditions, such as cell proliferation, apoptosis, cell cycle control, tumor promotion, and carcinogenesis (Shen et al., 2005). In addition, AP-1 controls the expression of a number of genes, such as those encoding collagenase, stromelysin, and cytokines through binding to its binding site (Shen et al., 2005). AP-1 is a heterodimeric complex of leucine-zipper proteins, c-Jun and c-Fos. Upon activation by a large number of stimuli, including proinflammatory cytokines, oxidative stress, and tumor promoters, AP-1 binds to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive elements (TRE) sequences within the promoter regions of several target genes (Shen et al., 2005).

AP-1 activity has been shown to be regulated by MAPK signaling pathways such as c-Jun NH_2-terminal kinase (JNK), extracellular-signal regulated kinase (ERK), and p38 MAPK (Shen et al., 2005). Once activated, JNK translocates to the nucleus where it phosphorylates c-Jun to potentiate its transcription activity which results in the induction of c-Jun and other AP-1 target gene transcription (Shen et al., 2005).

A well established link between the AP-1 signaling pathway and the expression of AhR-regulated genes was demonstrated previously (Yao et al., 1997; Suh et al., 2002). The role of AP-1 in the modulation of *CYP1A1* and *CYP1A2* gene expression is controversial. Several previous studies showed that TCDD inhibited LPS-induced DNA binding and transcriptional activity of AP-1 in murine lymphoma WT CH12.LX, but not in AhR-deficient BCL-1 cells (Suh et al., 2002). Furthermore, these results and the observations that AhR antagonists attenuated TCDD-induced inhibition of AP-1 binding in CH12.LX cells (Suh et al., 2002) strongly suggest a coordination between AhR and AP-1 signaling pathways. In contrast, treatment of Hepa 1c1c7 cells with TCDD or BaP caused an increase in c-Fos and c-Jun mRNA levels, which was associated with an increase of the DNA-binding activity of AP-1, suggesting that AP-1 activation requires a functional AhR-XRE complex (Hoffer et al., 1996). In addition, it has been shown that induction of CYP1A2 enzyme activity in HepG2 cells in response to 3MC is mediated through activation of AP-1 DNA binding (Quattrochi et al., 1998).

The involvement of AP-1 signaling pathways in the induction of *NQO1* gene expression has been previously reported. Several studies showed that induction of the *NQO1* gene in human colon adrenocarcinoma HT29 (Yao et al., 1997) and Hepa 1c1c7 (Park et al., 2004) cells by the chemoprotective compounds mitomycin C and baicalin is AP-1-dependent. Furthermore, it has been demonstrated that constitutive expression of *NQO1* in human non-small cell lung cancer is regulated through the AP-1 signaling pathway (Kepa and Ross, 1999).

1.5. TRACE METALS

Historically, metals are considered the oldest toxic substances known to humans. Probably, lead (Pb^{2+}) is probably the oldest metals having been used in 2000 B.C (Valko et al., 2005). In addition, the early use of arsenic and mercury (Hg^{2+}) was for the decoration of Egyptian tombs (Valko et al., 2005). Among metals, heavy metals are defined as the metallic elements that are capable of forming polyvalent cations and possess molecular size of more than 50 Da (Foulkes, 2000). Based on physiological and toxicological effects, heavy metals are classified into four classes (Foulkes, 2000). Class A represents heavy metals, such as iron, that play essential physiological functions such as mediation of enzyme activities. Class B includes heavy metals, such as strontium, that have no physiological role, but are slightly toxic in low-range concentrations. Metals that are essential for living systems, such as zinc, nickel, and copper (Cu^{2+}), belong to class C; however, they are considered very toxic at relatively high concentrations. Class D includes heavy metals that are very toxic at very low concentrations and have no biological functions, such as arsenite, Hg^{2+}, Pb^{2+}, and cadmium (Foulkes, 1990; Foulkes, 2000).

Heavy metals are highly toxic non-essential elements in that they are neither created nor biodegradable and usually originate from natural and industrial resourses (Barbier et al., 2005). The available information suggests that metals in the environment, even in trace amounts, can cause serious problems to all organisms including humans (Beyersmann, 2002). The frequent persistent occurrence and accumulation of heavy metals in the environment and their potential exposure to humans, from numerous sources, including

contaminated air, water, soil and food, cause them to be ranked highly as most hazardous and toxic substances in the environment by the Agency for Toxic Substances and Diseases Registry (ASTDR) (ATSDR, 2005) and the Canadian Environmental Protection Act Registry (CEPA) (CEPA, 2006). Among all heavy metals, Hg^{2+}, Pb^{2+}, and Cu^{2+} ranked the highest in these lists.

1.5.1. Hg

Hg is the only metal that is in a liquid state at room temperature. Hg exists in three different oxidation states. For example, Hg^0 usually exists in metallic form as a vapor, whereas Hg^+ or Hg^{2+} can form stable organic compounds such as methyl mercury. Generally, the route and efficiency of exposure depends mainly on the state of Hg. The primary sources of chronic Hg exposure are dental amalgams and fish consumption (Ercal et al., 2001; Tezel et al., 2001). Hg usually enters water as a product of industrial pollution, where is is methylated by bacteria to form methyl mercury, which then up taken by fish and ultimately by humans (Crinnion, 2000).

Liquid metallic Hg is slowly absorbed by the gastrointestinal tract, whereas the vapor form is rapidly absorbed into the lung and all other tissues in the body (Brambila et al., 2002). On the other hand, the mercurous form is poorly absorbed from the GIT. Studies in human volunteers showed that approximately 15% of ingested Hg is absorbed from the GIT, whereas the organic form, methyl mercury, is completely absorbed (>90%). Once reaching the blood circulation, it easily and rapidly crosses cell membranes. Inside the cell, Hg^0 is oxidized to its reactive form Hg^{2+} (Crinnion, 2000). The cell to plasma ratio

of the organic mercury is 10:1, whereas for inorganic form the ratio ranges from 1-2:1. While inorganic mercury accumulates in high concentration in the kidneys, brain, particularly the posterior cortex, contains the greatest concentrations of the organic and vapor forms. The excretion route of the mercury from the body depends on the form of the Hg, the size and time of exposure. For example, the inorganic form is initially eliminated via fecal excretion, whereas renal excretion increases with time (Brodkin et al., 2007). Organic mercury, however, is mainly excreted renally after acute or chronic exposure. The elimination half lives of mercury range from 20 to 90 days (Brodkin et al., 2007).

Although the signs and symptoms of mercury toxicity depend on the form of mercury and the route of exposure, gingivitis, stomatitis, tremor, and fatigue are early common manifestations of Hg toxicity (Brodkin et al., 2007). In adults, these signs and symptoms begin to occur at blood levels of approximately 0.5 μM (Brodkin et al., 2007). In addition, severe hepatotoxicity, nephrotoxicity, neurotoxicity, and immunotoxicity are common toxicities of Hg (Crinnion, 2000)

1.5.2. Pb

Pb is a ubiquitous toxic heavy metal that is not required by the body and is considered toxic to living organisms even in very small traces. Pb exists in the environment either in a solid form as dust, or particulates, or in a vapor form (ATSDR, 2005; Brodkin et al., 2007). Upon exposure to Pb^{2+} from different sources, such as food, lead-based indoor paints, contaminated leaded water, and leaded gasoline, more than 90% of inhaled Pb

particulates are retained in the alveoli and hence absorbed completely into the circulation. Once in the blood, Pb mostly targets red blood cells and hemoglobin. Blood Pb is in equilibrium between plasma and erythrocytes in a ratio of 1:100 (Manton et al., 1984).

Two kinetic pools for Pb have been demonstrated. The largest and slowest kinetic pool is the disposition of lead into the bone and the skeleton, with a $t_{1/2}$ of more than 20 years (Silbergeld et al., 1988). However, the bone lead is not an inert storage site for absorbed lead. In fact, several experimental studies have suggested that lead can be released from the bone during conditions of demineralization, such as pregnancy and lactation (Silbergeld et al., 1988). Absorbed Pb is excreted mainly through the kidneys, and to some extent through other body fluids such as breast milk (Gulson et al., 1998a; Gulson et al., 1998b).

The toxic effects of prolonged and chronic Pb exposure involve various organ systems and physiological activities (Brodkin et al., 2007). Epidemiological studies showed that adults with excess occupational or accidental exposure with blood Pb levels of approximately 40 μg/dl demonstrated abnormalities in a number of neurobehavioral measurements, such as slower response for pattern memory (Moore et al., 1986; Payton et al., 1998). Possible mechanisms include changes in the activities of several enzymes such as tyrosine kinase and choline acetyltransferase (Moore et al., 1986; McIntosh et al., 1989).

1.5.3. Cu

Cu^{2+} is a naturally distributed and nutritionally essential element. The daily allowance intake of Cu in adults ranges between 0.9-2.2 mg, but decreases to 0.6-0.8 mg in children (WHO, 1998). The normal serum level of Cu^{2+} is approximately 0.12-1.45 mg/L. The World Health Organization (WHO) has estimated a 1.3 mg/L level of Cu^{2+} in drinking water as a contaminant.

Upon exposure, 40% of ingested Cu^{2+} is absorbed from the small intestine, through an amino acid transport system for amino acids such as histidine, methionine, and cysteine, into the blood (Valko et al., 2006; Valko et al., 2007). Cu^{2+} in the blood binds initially to albumin and later more firmly to ceruloplasmin and transcuprein, which represents about 80% of the total Cu^{2+} circulating in the blood. Cu^{2+} is then up taken by and stored in hepatocytes predominantly complexed to GSH prior to binding with metallothionein. The hepatic Cu^{2+}-metallothionein complex is mainly excreted via biliary excretion (Mattie and Freedman, 2004). Several studies in human subjects showed that ingestion of large amounts of Cu^{2+} exceeding 3 mg/L caused gastrointestinal symptoms, hepatic failure, Wilson's diseases, and death in some cases. The ability of Cu^{2+} to increase cellular formation of ROS and oxidative stress is one of the mechanisms by which Cu^{2+} induces toxicities.

1.6. RATIONALE, HYPOTHESES, AND OBJECTIVES

1.6.1. Rationale

Co-contamination with complex mixtures of trace metals and AhR ligands is a common environmental problem with multiple biological consequences, particularly to the metabolizing enzyme systems. Both AhR ligands and trace metals are ranked highly on the list of the most hazardous xenobiotics in the environment, prepared by the Canadian Environmental Protection Act (CEPA, 2006). The AhR plays a central role in the toxicity of PAHs and HAHs by controlling the expression of a battery of enzymes responsible for their metabolism. The toxicity of AhR ligands is based on their bioactivation by CYP1A1 to yield mutagenic and carcinogenic intermediates, which are then detoxified via induction of NQO1 and GSTA1 genes.

Many sources of environmental exposure to AhR ligands involve trace metals co-exposure. Trace metals and AhR ligands are common contaminants of hazardous waste sites and co-released from sources such as fossil fuel combustion and municipal waste incineration and as components of tobacco. Several studies have reported that significant traces of metals were detected in the air of several cities in Italy, Pakistan, and the Czech Republic (Sram et al., 1996; Monarca et al., 1997), in surface soils in Greece (Stalikas et al., 1997) in Sweden, and in fish from the Sheboygan River in Wisconsin (Kaminsky, 2006). In Canada, a study examined trace metal levels in scalp hair taken from 122 children and 27 adult liveing in Alberta showed that Pb^{2+}, Cu^{2+}, nickel and cadmium were the highest in adults (Moon et al., 1986; Moon et al., 1988).

Several previous studies have addressed trace metals as modifiers of xenobiotic metabolizing enzymes, suggesting a possible role of trace metals in altering the mutagenicity and carcinogenicity of PAHs and HAHs. Among these metals, Hg^{2+}, Pb^{2+}, and Cu^{2+} are ranked highly among the most hazardous and toxic substances in the environment (ATSDR, 2005; CEPA, 2006). However, very little information is available on the effects of Hg^{2+}, Pb^{2+} and Cu^{2+} on the regulation and expression of AhR-regulated genes. Initial reports on the effects of Hg^{2+} on CYPs activity have demonstrated that Hg^{2+} decreased hepatic CYPs in both phenobarbital- and 3MC-treated mice (Abbas-Ali 1980). In addition, Hg^{2+} has been shown to enhance the activity of NQO1 and catecholamine-O-methyl-transferase in human placenta (Boadi *et al.*, 1991). In rats, Pb^{2+} significantly decreased CYP1A1 and CYP1A2 at the mRNA, protein, and activity levels (Degawa *et al.*, 1995), whereas all GST subunits in rat liver and kidney were increased, which was associated with an increase in lipid peroxidation (Daggett *et al.*, 1998). In addition, it has been reported that Cu^{2+} deficiency increases EROD activity in rat small intestine (Johnson and Smith, 1994).

1.6.2. Hypotheses

1) Co-exposure to metals and AhR ligands disrupts the expression of AhR-regulated genes, 2) Trace metals modulate the expression of AhR-regulated genes at the transcriptional and post-transcriptional levels through AhR-dependent mechanisms, and 3) Redox-sensitive transcription factors such as Nrf2, NF-κB, and AP-1 are involved in the modulation of AhR-regulated genes by metals.

1.6.3. Objectives

The specific objectives of the present work are: 1) to determine the capacity of the three prominent environmental metal contaminants, Hg^{2+}, Pb^{2+} and Cu^{2+}, to affect the induction of *Cyp1a1*, *Nqo1*, and *Gsta1* genes by four potential AhR ligands such TCDD, 3MC, βNF, and BaP, 2) to examine the molecular mechanisms by which metals modulate the expression of AhR-regulated genes, and 3) to characterize the role of oxidative stress and redox-sensitive transcription factors, Nrf2, NF-κB, and AP-1 in the modulation of *Cyp1a1*, *Nqo1*, and *Gsta1* genes by metals.

The importance and the significance of this research project derives from its great impact on increasing our knowledge about the effects of metals on the AhR-regulated genes. This will allow us to understand and identify the mechanistic steps and signaling pathways likely to be involved in the modulation of xenobiotic metabolizing enzymes by metals, which will help in improving the treatment strategies for mutagenicity medited by AhR ligands.

CHAPTER 2- MATERIALS AND METHODS

2.1. CHEMICALS

Lead nitrate, mercuric chloride, benzo[a]pyrene (BaP), 3-methylcholanthrene (3MC), β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), bovine serum albumin, cumene hydroperoxide (CHP), 1-chloro-2,4-dinitrobenzene (CDNB), 2′,7′-dichlorofluorescein diacetate (DCF-DA), Dulbecco's Modified Eagle's Medium (DMEM), 7-ethoxyresorufin (7ER), fluorescamine, glucose, reduced glutathione, glutathione reductase, 2,6-dichlorophenolindophenol (DCPIP), dihydrorhodamine-123 (DHR-123), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 7-methoxyresorufin (7MR), protease inhibitor cocktail, lipopolysaccharide (LPS), L-buthionine-(S,R)-sulfoximine (BSO), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), 1,9-pyrazoloanthrone (SP600125), (1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene ethanolate (U0126), phorbol 12-myristate 13-acetate (PMA), pyrrolidinedithiocarbamate (PDTC), cycloheximide (CHX), diethyl pyrocarbonate (DEPC), polyvinylpyrrolidone, ficoll, sheared salmon sperm DNA, phenylmethanesulphonylfluoride (PMSF), and anti-goat IgG peroxidase secondary antibody were purchased from Sigma Chemical Co. (St. Louis, MO). 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD), >99% pure, was purchased from Cambridge Isotope Laboratories (Woburn, MA). Tris hydrochloride, agarose, formamide and sodium azide were purchased from EM Science (Gibbstown, NJ). Tween-20 was purchased from BDH Inc. (Toronto, ON). Cupric sulfate, amphotericin B, resorufin, β-naphthoflavone (βNF), dithiothreitol (DTT), and 100× vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, QC). Gentamicin sulfate, penicillin–strepromycin, L-glutamine, MEM non-essential amino acids solution, fetal

bovine serum, TRIzol reagent, T4 polynucleotide kinase, and the random primers DNA labeling system were purchased from Invitrogen Co. (Grand Island, NY). Hybond-N-nylon membranes, poly(dI.dC), and chemiluminescence Western blotting detection reagents were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). α-^{32}P-dCTP and γ-^{32}P-ATP (3000 Ci/mmol) were supplied by DNA Core Services Laboratory University of Alberta (Edmonton, AB). Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, *N'N'*-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose membrane (0.45 μm), sodium dodecyl sulfate (SDS), and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1 goat anti-mouse polyclonal primary antibody (G-18), rabbit Nrf2 polyclonal primary antibody, and anti-rabbit IgG peroxidase secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat GSTA1 anti-rat polyclonal primary antibody was purchased from Oxford Biomedical Research (Oxford, MI). Rabbit NQO1 anti-human polyclonal primary antibody was generously provided by Dr. David Ross, University of Colorado Health Sciences Center (Denver, CO). Skim milk was obtained from DIFCO Laboratories (Detroite, MI). Actinomycin D (Act-D) and carbobenzoxy-L-leucyl-L-leucyl-leucinal (MG-132) were purchased from Calbiochem (San Diego, CA). All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

2.2. METHODS

2.2.1. Cell Model

To test the hypotheses raised in this proposal, murine Hepatoma Hepa 1c1c7 cell lines were utilized. Hepa 1c1c7 cell lines were first established in early 1970s from the transplantable hepatoma BW 7756 originally produced in a C57L mouse and propagated in C57L/J mice (Bernhard et al., 1973). Hepa 1c1c7 cell lines have been extensively used to elucidate pathways and mechanisms involving the AhR (Maier et al., 2000; Bonzo et al., 2005; Kann et al., 2005).

Hepa 1c1c7 cells represent a powerful *in vitro* experimental model for investigating the mechanisms by which metals influence the regulation of the AhR-regulated genes for the following reasons. First, basal and inducible levels of AhR-regulated genes in these cells are much greater than those found in rat or human cell lines (Benedict et al., 1973). Therefore, with the use of metals, chemical inhibitors, activators, or combinations, suitable changes in the expression of these genes can be measured with confidence. Second, the hepatocyte has been shown to be one of major targets for metals upon exposure; several studies on metal toxicity have demonstrated high uptake and accumulation of these toxic metals in the liver (Ercal et al., 2001). Third, the availability of a mutant Hepa 1c1c7 cell line deficient in AhR allows the study of events in this signaling cascade that are dependent on the presence of AhR. The AhR deficient Hepa 1c1c7 C12 cells exhibit a 90-95% decreased responsive to TCDD as demonstrated by TCDD binding affinity and Western blot analysis (Hankinson et al., 1985; Ma and Whitlock, 1996; Hankinson, 2005). The AhR concentrations in these cells decreased to

less than 1 fmol/10^6 cells compared to approximately 20 fmol/10^6 in WT cells (Legraverend et al., 1982). Yet no strains with a complete absence of AhR have been identified, suggesting that a very finite level of AhR is required for Hepa 1c1c7 cell survival.

2.2.2. Cell Culture

Murine hepatoma Hepa 1c1c7 WT and C12 cells, generously provided by Dr. Oliver Hankinson, University of California (Los Angeles, CA), were maintained in standard DMEM media without phenol red and supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamicin sulfate, 100 IU/ml penicillin G, 10 μg/ml streptomycin, 25 ng/ml amphotericin B, 0.1 mM MEM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm^2 tissue culture flasks at 37°C under a 4% CO_2 humidified environment. The medium was changed every 2 days and the cells were subcultured every 3 days at a 1:3 ratio.

2.2.3. Chemical Treatments

For the Cyp1a1 enzyme activity assay, Hepa 1c1c7 cells were seeded at a cell density of 7.5×10^4 cells/well in flat bottom with low evaporation lid 96-well cell culture plates, and plated at a cell density of 1.5×10^6 cells/well in six-well cell cultured plates for the Nqo1 and Gsta1 enzyme activity assays. For the RNA assay, cells were grown in six-well cell culture plates in a DMEM culture media. The cells were treated in serum-free media with AhR ligands, metals, or combinations, in which various metals were added 30 min prior to the addition of AhR ligands, when applicable. For experiments using signaling

pathway modulators, Hepa 1c1c7 cells were pretreated for indicated time intervals in serum-free media with various chemical inhibitors or activators prior to the addition of metals and/or AhR ligands as indicated, wherever applicable. Stock solutions of metals (10 mM) were freshly prepared in sterile double de-ionized water followed by serial dilutions just prior to treatment. Stock solutions of AhR ligands, PMA, U0126, SP203580, MG-132, and SP600125 were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C; the concentrations of DMSO did not exceed 0.05% (v/v). On the other hand, stock solutions of PDTC, CHX, and BSO were freshly prepared in sterile double de-ionized water just prior to treatments.

2.2.4. Measurement of Cell Viability

The MTT assay was performed to determine the maximum non-toxic concentrations of each metal to be used in the current study as described previously (Vakharia et al., 2001b). Viability of Hepa 1c1c7 WT and C12 cells after treatment with the test compounds was determined by examining the capacity of reducing enzyme present in viable cells to convert MTT to formazan crystals. Twenty-four hours after incubating the cells with the test compounds in a 96-well cell culture plate at 37 °C under a 4% CO_2 humidified incubator, the media were removed and a 100 µl of serum-free medium containing 1.2 mM of MTT dissolved in PBS, pH 7.2, was added to each cell culture well. The plate was then incubated in a CO_2 incubator at 37 °C for 2 h. The media were then decanted off by inverting the plate and 00 µl of isopropyl alcohol was added to each well, with shaking for 1 h to dissolve the formazan crystals. The color intensity in each well was measured at wavelength of 550 nm using a BIO-TEK Instruments EL 312e

microplate reader, Bio-Tek Instruments (Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.2.5. Determination of Cyp1a1 Enzyme Activity

EROD assays were performed on intact living Hepa 1c1c7 WT and C12 cells using 7ER as a substrate (Kennedy et al., 1993). After incubation of the cells with test compounds for 24 h in 96-well cell culture plates, media were aspirated and the cell monolayers were rinsed with warmed (37° C) PBS. Thereafter, 100 µl of 2 µM 7ER in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8) that had been prewarmed to 37° C, was then added to each cell well. Immediately, an initial fluorescence measurement (t=0) at excitation/emission (545 nm/575 nm) was recorded from each cell well using a Baxter 96-well fluorometer (Deerfield, IL). The plates were then replaced in the incubator, and an additional set of fluorescence measurements of the cell wells was recorded every 5 min for 20 min interval. The amounts of resorufin formed in each well weredetermined by comparison with a standard curve of known concentrations. The working solution was then aspirated, the cell wells were rinsed twice with PBS, and 50 µl of double de-ionized water was added to lyse the cells. After placing the cell plates at -80°C for 30 min, the cell lysates were allowed to thaw, and protein levels were determined using a modified fluorescent assay (Lorenzen and Kennedy, 1993). The rate of resorufin formation was expressed as pmol/min/mg protein.

2.2.6. Preparation of Cell Homogenate

Twenty-four hours after incubating the cells with treatments in six-well cell culture plates, cultured cells were washed with phosphate buffered saline (PBS) and then 0.5 ml of homogenization buffer (50 mM potassium phosphate, pH 7.4, and 1.15% KCl) was added to each well. The plates were then incubated for 24 h in a −80 °C freezer. Thawed cells were extracted and homogenized using a Kontes homogenizer at 4 °C before they were centrifuged at 10,000×*g* for 20 min. The supernatant fractions were collected for determination of protein concentration using bovine serum albumin as a standard by the Lowry method (Lowry et al., 1951). The extracted cytosolic fractions were stored in a −80 °C freezer for later use in the determination of the Nqo1 and Gsta1 enzyme activities.

2.2.7. Determination of Nqo1 Enzyme Activity

Nqo1 activity was determined by quantitation of the reduction rate of its substrate DCPIP using the continuous spectrophotometric assay of Ernster (Ernster et al., 1962; Ernster et al., 1972). Approximately 10 μg cell homogenate protein was incubated with 1 ml of the assay buffer (40 μM DCPIP, 0.2 mM NADPH, 5 μM FAD, 25 mM Tris–HCl, pH 7.8, 0.1% (v/v), Tween-20, and 0.023% bovine serum albumin), and the rate of DCPIP reduction was monitored over 1.5 min at 600 nm with an extinction coefficient (ε) of 2.1 mM^{-1} cm^{-1}. Nqo1 activity was calculated as the decrease in absorbance per min per mg total protein of the sample.

2.2.8. Determination of Gsta1 Enzyme Activity

Spectrophotometric assays for Gsta1 enzyme activity using CHP as a substrate were conducted according to the method of (Beaumont et al., 1998). These reactions were performed in 0.1 M potassium phosphate buffer, pH 7.0, containing sodium azide, at 25 °C in a total volume of 1 ml. The rate of the enzyme-catalyzed reaction was determined by subtracting the rate of reaction occurring in the absence of glutathione reductase. Approximately 200 µg cell homogenate protein was incubated with 0.5 mM CHP, 1 mM reduced glutathione, 0.1 mM NADPH, and 0.3 unit of glutathione reductase. The rate of NADPH disappearance was monitored for 1.5 min at 340 nm with ε=6.2 mM^{-1} cm^{-1}. The Gsta1 activity was calculated as the decrease in absorbance per min per mg total protein of the sample.

2.2.9. Measurement of Cellular Heme Content

Cellular heme content was determined fluorimetrically as described previously (Ward et al., 1984). Hepa 1c1c7 cells were plated in 100 mm petri dishes for 48 h and thereafter incubated in fresh serum-free medium with test compounds for 24 h. Cells were then collected in BPS by scraping into 1.5 ml tubes and centrifuged at 2,000 x *g* for 1 min. Pelleted cells were boiled in 2 M oxalic acid (100 µl) for 30 min and then rapidly resuspended in cold PBS (0.9 ml) followed by centrifugation at 14,000 x *g* for 15 min. The supernatant was assayed for protoporphyrin IX using Eclipse fluorescence spectrophotometer, Varian Inc., (Palo Alto, CA) with excitation and emission wavelengths of 405 and 600 nm, respectively. Cellular heme content was normalized for cellular protein which was determined using Lowry method (Lowry et al., 1951).

for 15 min at 2 to 8°C. The aqueous phase which contains RNA was then transferred to a fresh tube and 0.3 ml isopropyl alcohol was then added to each tube to precipitate the RNA by freezing the samples at – 20 C for 2 h. Following centrifugation at 12,000 x *g* for 10 min at 2 to 8°C, the RNA pellet was washed once with 75% ethanol in DEPC-treated water, dried, and then dissolved in DEPC-treated water. RNA was quantified by measuring the absorbance at 260 nm.

Northern blot analysis of total RNA was performed as described elsewhere (Sambrook et al., 1989). Briefly, aliquots of 20 μg of RNA were separated in a denaturing (0.22 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N-nylon membranes. The RNA was cross-linked to the membranes using the UV Stratalinker 2400 (Stratagene, La Jolla, CA), followed by baking at 80 °C for 3 h. Prehybridization of the membranes was carried out in a solution containing: 6× SSPE (0.9 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.02 M EDTA); 50% deionized formamide; 5× Denhardt's reagent (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin); 0.5% SDS; and 100 μg/ml sheared salmon sperm DNA, for at least 4 h at 42 °C. Hybridization with the [^{32}P]-labeled cDNA probes was carried out in the same solution, without Denhardt's reagent, for 16–24 h at 42 °C. The membranes were then washed twice at room temperature in a solution containing 2× SSPE and 0.5% SDS for 5 min for the first wash, and 15 min for the second wash. This was followed by a 30 min wash in 0.1× SSPE, 0.5% SDS at 42 °C and a final 30 min wash in 0.1× SSPE, 0.5% SDS at a temperature of 62 °C for 30 min. The washed membranes were rinsed in a 0.1× SSPE solution, dried, sealed in plastic wrap and visualized by exposure to Kodak BioMax MS film. Hybridization signals were quantified

relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA by dividing the level of Nqo1 or Gsta1 mRNA by the level of Gapdh mRNA in the same sample, and the results were expressed as a percentage of the control values taken as 100%, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

The cDNA probes for mouse Cyp1a1, HO-1, and Gapdh mRNAs were generously provided by Dr. John R. Bend, University of Western Ontario (London, ON). The cDNA probe for mouse Gsta1 mRNA was provided by Dr. David Eaton, University of Washington (Seattle, WA). The mouse Nqo1 cDNA probe was prepared from previously described primer sequences (Chen et al., 1994) by reverse transcription coupled to polymerase chain reaction (PCR) amplification using the primers FNAD(P)H:QOR 5′-GCCATGGCGGCAGAAGAGCCCTG-3' (F1; forward) and BNAD(P)H:QOR 5′-CTTATTTTCTAGCTTTGATCTG-3′ (R1; reverse) containing Nco 1 and Hind III restriction sites, respectively. All probes were [^{32}P]-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions. The intensities of mRNA bands were quantified, relative to the signals obtained for Gapdh mRNA, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

2.2.12. Protein Extraction and Western Blot Analysis

Twenty-four hours after incubating the cells with test compounds, the cells were washed once with cold PBS and collected by scraping in 100 µl of lysis buffer (50 mM HEPES,

0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µl/ml of protease inhibitor cocktail). The lysates were incubated on ice for 1 h with intermittent vortexing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was then stored at a −80 °C freezer for later use in the Western blot analysis.

Western blot analysis was performed as described previously (Sambrook et al., 1989). For Cyp1a1 immunodetection, approximately 10 µg of proteins from each treatment group were diluted with same amount (1:1) of 2X loading buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 1.5% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol), boiled and loaded onto a 7.5% SDS-polyacrylamide gel. Samples were electrophoresed at 120 V for 2 h, and separated proteins were transferred to Trans-Blot nitrocellulose membrane (0.45 µm) in a buffer containing 25 mM Tri–HCl, 192 mM glycine, and 20% (v/v) methanol. Protein blots were blocked overnight at 4 °C in a solution containing 5% skim milk powder, 2% bovine serum albumin and 0.5% Tween-20 in TBS solution (0.15 M NaCl, 3 mM KCl, 25 mM Tris-base). Thereafter, the blocking solution was removed and the blots were rinsed three times in a wash buffer (0.1% Tween-20 in TBS). Proteins were detected by incubation with a primary polyclonal goat anti-mouse CYP1A1 antibody (G-18) for 2 h at 4 °C in TBS containing 0.01% sodium azide and 0.05% Tween-20. The primary antibody solution was removed and blots were rinsed three times with a wash buffer, followed by incubation with horseradish peroxidase-conjugate rabbit anti-goat secondary antibody for 1 h at room temperature followed by washing as

previously described. Antibody detection was performed using the enhanced chemiluminescence method.

For Nrf2 immunodetection, 75 μg of protein from each treatment group were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked overnight at 4 °C, followed by incubation with primary antibody against Nrf2 for 3 h at room temperature, and then 1 h incubation with a peroxidase-conjugated anti-rabbit secondary antibody at room temperature. For Nqo1 immunodetection, 200 μg of protein from each treatment group were separated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked overnight at 4 °C, followed by incubation with rabbit anti-human polyclonal primary antibody against Nqo1 overnight at 4 °C, and then 2 h incubation with a peroxidase-conjugated anti-rabbit secondary antibody at room temperature. Nqo1 blots were subsequently stripped in a solution containing 62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 50 °C, and then re-probed with goat anti-rat primary antibody against Gsta1 overnight at 4 °C. A horseradish peroxidase-conjugate rabbit anti-goat secondary antibody was the incubated for 1 h at room temperature followed by washing as previously described. All bands were visualized using enhanced chemiluminescence method according to the manufacturer's instructions, GE Healthcare (Little Chalfont, Buckinghamshire, UK) and quantified relative to the signals obtained for β-actin, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

12,000 × g for 20 min at 4 °C. The resulting supernatant was then centrifuged at 100,000 × g for 1 h at 4 °C to obtain the final nuclear protein extract.

Because of low efficiency of transformation of the mouse AhR, due to the extreme resistance of HSP90 to dissociate from the mouse AhR, while the greatest degree of transformation of the guinea pig AhR in response to AhR ligand (Bohonowych and Denison, 2007), we used guinea pig cytocol as a model. Therefore, hepatic cytosol of untreated guinea pig [generously provided by Dr. Michael S. Denison, University of California (Davis, CA)] was prepared as described previously (Rogers and Denison, 2002) and then was incubated *in vitro* with the test compounds for 3 h.

2.2.16. Electrophoretic Mobility Shift Assay (EMSA).

XRE complementary oligonucleotides, 5-GGAGTTGCGTGAGAAGAGCC-3 and 5-GGCTCTTCTCACGCAACTCC-3, were synthesized, then annealed by heating to 70°C for 7 min, then allowed to cool to room temperature. The double-stranded XRE was then labeled with γ-^{32}P-ATP at the 5-end using T4 polynucleotide kinase (Invitrogen), according to the manufacturer's instructions, and used as a probe for EMSA reactions. EMSA was performed as described previously (Rogers and Denison, 2002). Briefly, aliquots of the nuclear extract (10 µg) or cytosolic protein (80 µg) were incubated for 30 min at room temperature in a reaction mixture (30 µl) containing 25 mM HEPES, pH 7.9, 80 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), 1 µg salmon sperm DNA, and 5 µg poly(dI.dC). Thereafter, ~1 ng (100,000 cpm) γ-^{32}P-labeled XRE was incubated with the mixture for another 30 min before being separated through a 4% nondenaturing

PAGE. For the competition assay, nuclear or cytosolic proteins were preincubated at room temperature for 30 min with a 100-fold molar excess of unlabeled XRE before the addition of the ^{32}P-labeled XRE. The gel was dried at 80°C for 1h, and then visualized by autoradiography.

2.2.17. Preparation of Nuclear Extracts for NF-κB and AP-1 EMSA.

Nuclear extracts from Hepa 1c1c7 cells were prepared according to a previously described procedure (Andrews and Faller, 1991) with slight modifications. Briefly, Hepa 1c1c7 cells grown on 100 mm petri dishes were treated for 2 h in a serum-free media with the test compound. Thereafter, cells were washed twice in cold PBS, pelleted and suspended in cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mm $MgCl_2$, 10 mM KC1, 0.5 mM DTT, and 0.2 mM PMSF). After 10 min on ice, the cells were centrifuged and the pellets were suspended again in cold buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). The cells were then incubated on ice with vigorous agitation for 30 min followed by a centrifugation for 10 min at 12000 *g* at 4° C. The nuclear extracts (supernatant) were stored at -80° C.

2.2.18. NFκB and AP-1 EMSA.

Oligonucleotides of κB (5- GGCAGGGGAATTCCCC-3) and TRE (5-GATCTGCATGAGTCAGACA-3) were synthesized and self-annealed as described previously (Puga et al., 2000; Ke et al., 2001). The oligonucleotides were then labeled with [γ-^{32}P]ATP at the 5-end using T4 polynucleotide kinase to be used as probes for

EMSA, according to the manufacturer's instructions (Invitrogen). Approximately 5-10 μg nuclear extract proteins were incubated for 30 min at room temperature in a binding reaction mixture (20 μl) containing 20 mM HEPES, pH 7.9, 40 mM KCl, 1 nM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol (v/v), 2 μg poly(dI.dC), and ~30,000 cpm [^{32}P]-labeled oligonucleotides. In order to confirm DNA binding specificity, nuclear extracts proteins were preincubated at room temperature for 10 min with a 100-fold molar excess of unlabeled probes before the addition of the [^{32}P]-labeled oligoneucleotides. The reaction mixtures were then separated through a 4% nondenaturing PAGE, and the gel was dried at 80°C for 30-45 min, and then visualized by autoradiography.

2.2.19. Statistical Analysis.

All results are presented as mean ± SEM. The comparative analysis of the results from various experimental groups with their corresponding controls was performed using SigmaStat for Windows, Systat Software Inc., (San Jose, CA). One-way analysis of variance (ANOVA) followed by Student–Newman–Keul's test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when $p<0.05$.

CHAPTER 3 - RESULTS

3.1. EFFECTS OF Hg^{2+}, Pb^{2+}, and Cu^{2+} ON THE EXPRESSION OF AhR-REGULATED GENES

3.1.1. Effect of metals in the absence and presence of AhR ligands on Hepa 1c1c7 cell viability

To investigate the effects of metals in the absence and presence of AhR ligands on WT and C12 Hepa 1c1c7 cell viability and hence to determine the maximum non-toxic concentrations to be used in the current study, an MTT assay was performed. For this purpose, cells were treated for 24 h with a wide range of concentrations from each metal, Hg^{2+} (2.5–10 μM), Pb^{2+} (10–100 μM), and Cu^{2+} (1–100 μM), in the absence and presence of a single concentration of an AhR ligand, TCDD (0.1 nM), 3MC (0.25 μM), BaP (1 μM), or βNF (10 μM).

Figure 3.1A shows that neither metal alone nor metals plus AhR ligand mixture at all concentrations tested were toxic to WT cells in which cell viability was not significantly altered (≥90%); the exception was Hg^{2+}, which decreased cell viability by approximately 20% in the presence of TCDD. On the other hand, C12 cells were generally more sensitive to the metals (Fig. 3.1B). Cu^{2+} was the most toxic, with about 40–60% decreases in cell viability observed at the highest concentration tested, 100 μM, in the absence and presence of AhR ligands. The sensitivity of C12 cells to metals compared to WT could be attributed, at least in part, to the facts that these mutant cells (C12) exhibite higher mitochondrial activity associated with an increase in cytochrome c oxidase basal expression (Fong et al., 2005).

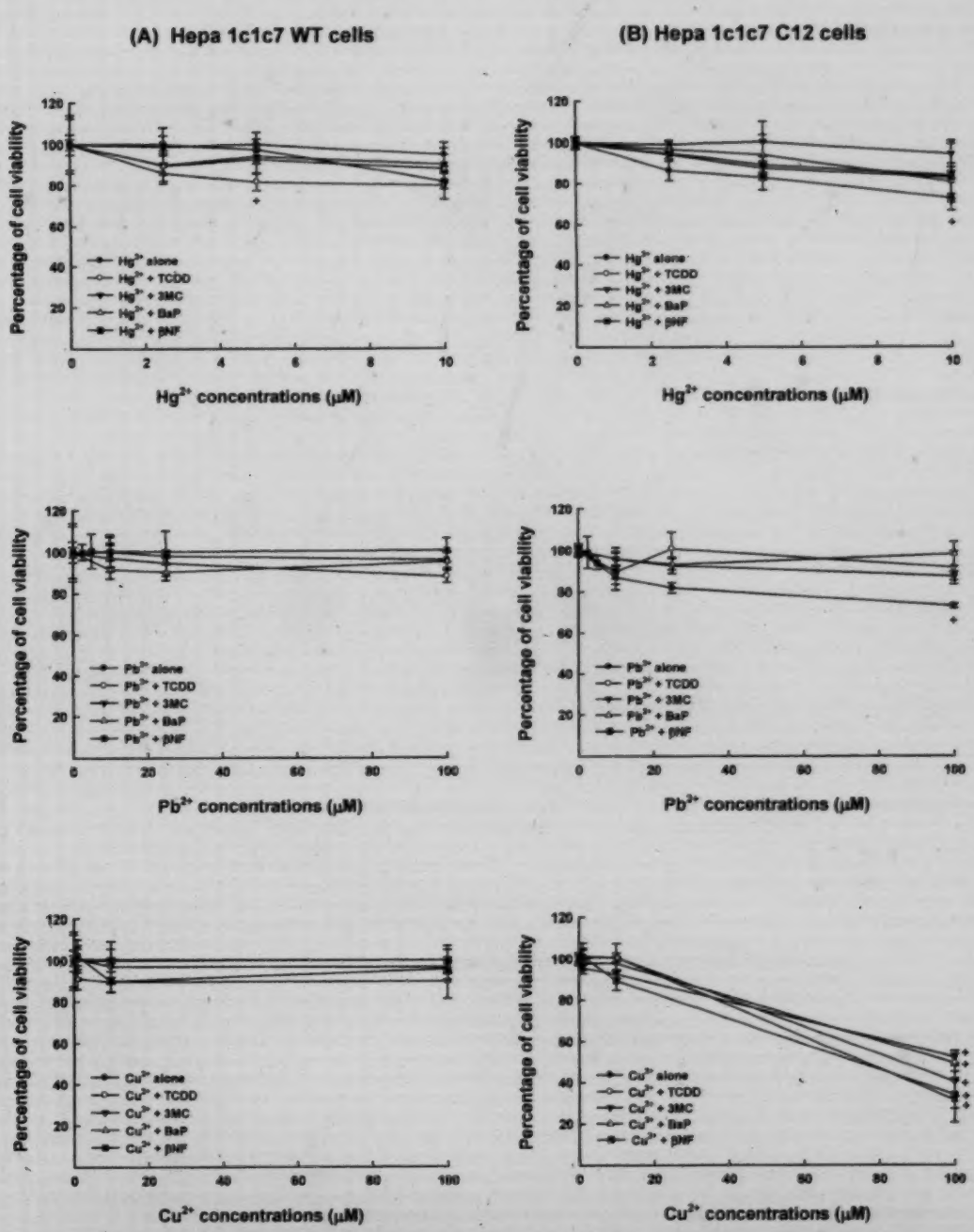

Figure 3.1. Effect of metals on cell viability. (A) Hepa 1c1c7 WT and (B) C12 cells were treated for 24 h with various concentrations of Hg^{2+} (2.5, 5 and 10 μM), Pb^{2+} (10, 25 and 100 μM), and Cu^{2+} (1, 10 and 100 μM) in the absence and presence of AhR ligands, TCDD (0.1 nM), 3MC (0.25 μM), BaP (1 μM), or βNF (10 μM). Cell viability was determined using MTT assay. Values are presented as percent of the control (mean ± SEM, n = 6). $^{+}p < 0.05$ compared with control.

3.1.2. Effect of Metals on Constitutive and Inducible Cyp1a1 Activity in WT Cells

EROD activity is primarily catalyzed by CYP1A1 and thus is a useful biomarker for the effect of metals as well as AhR ligands on CYP1A1-dependent catalytic activity (Burke et al., 1994). Very little information is available regarding the effects of Hg^{2+}, Pb^{2+}, and Cu^{2+} on Cyp1a1-catalytic enzyme activity, particularly, in Hepa 1c1c7 cells. Therefore, in this study, we investigated the effects of metals on the constitutive and inducible EROD activity in Hepa 1c1c7 WT cells. The cells were treated with increasing concentrations of Hg^{2+}, Pb^{2+}, and Cu^{2+} in the absence and presence of AhR ligands. Our data showed that metals alone, at the concentrations tested in this study, did not significantly alter the constitutive EROD activity in Hepa 1c1c7 WT cells. On the other hand, as expected, all AhR ligands used in this study significantly induced Cyp1a1 activity (Fig. 3.2). The order of potency of induction of Cyp1a1 activity by AhR ligands, at the concentrations tested, was βNF>3MC>TCDD>BaP.

To investigate the potential interactions between metals and AhR ligands on Cyp1a1 catalytic activity, WT cells were simultaneously exposed to increasing concentrations of metals and a single dose of each AhR ligand that have been shown to cause submaximal induction of Cyp1a1 enzyme activity to determine whether metal treatment would potentiate or inhibit the induction of Cyp1a1 by AhR ligands. All metals tested in this study showed a variable capacity to alter the AhR ligands-mediated induction of Cyp1a1 activity. All three metals decreased the induction of Cyp1a1 catalytic activity by AhR ligands in a concentration-dependent manner (Fig. 3.2). The maximum inhibitory effect of metals on the induction of Cyp1a1 activity was observed with Cu^{2+}, followed by Pb^{2+}

and then Hg^{2+}. Interestingly, we have found that BaP-mediated induction of Cyp1a1 activity was the least sensitive to the inhibitory effect of Hg^{2+}, whereas Hg^{2+} exhibited the maximum inhibitory effects on βNF-induced Cyp1a1 activity (Fig. 3.2A).

3.1.3. Effect of Metals on Constitutive and Inducible Nqo1 and Gsta1 Activities in WT Cells

To determine the effect of metals on the constitutive and inducible Nqo1 and Gsta1 activities, WT cells were exposed for 24 h to a range of metal concentrations with or without AhR ligands. Nqo1 and Gsta1 activities were differently affected by metals. All metals alone significantly increased the Nqo1 activity in a dose-dependent manner, in which the order of potency of Nqo1 induction by metals was $Cu^{2+}>Hg^{2+}>Pb^{2+}$ (Fig. 3.3). With respect to Gsta1, Hg^{2+} and Pb^{2+} at lower concentrations (2.5 and 10 μM, respectively) increased the Gsta1 activity by approximately 13% ($P>0.05$) and 36% ($P<0.05$), respectively, while at higher concentrations, Gsta1 activity was significantly decreased (Fig. 3.4A and B). Cu^{2+}, on the other hand, increased the Gsta1 activity in a dose-dependent manner by nine-fold at the highest concentration tested (Fig. 3.4C).

In the current study, we found that Nqo1 was more sensitive than Gsta1 to the induction by AhR ligands. All AhR ligands significantly induced Nqo1 activity as shown in Fig. 3.3, but failed to induce Gsta1 at the concentrations tested, with the exception of βNF which significantly increased the Gsta1 activity by 37% (Fig. 3.4). The order of potency of induction of Nqo1 by AhR ligands was βNF>3MC>BaP>TCDD (Fig. 3.3).

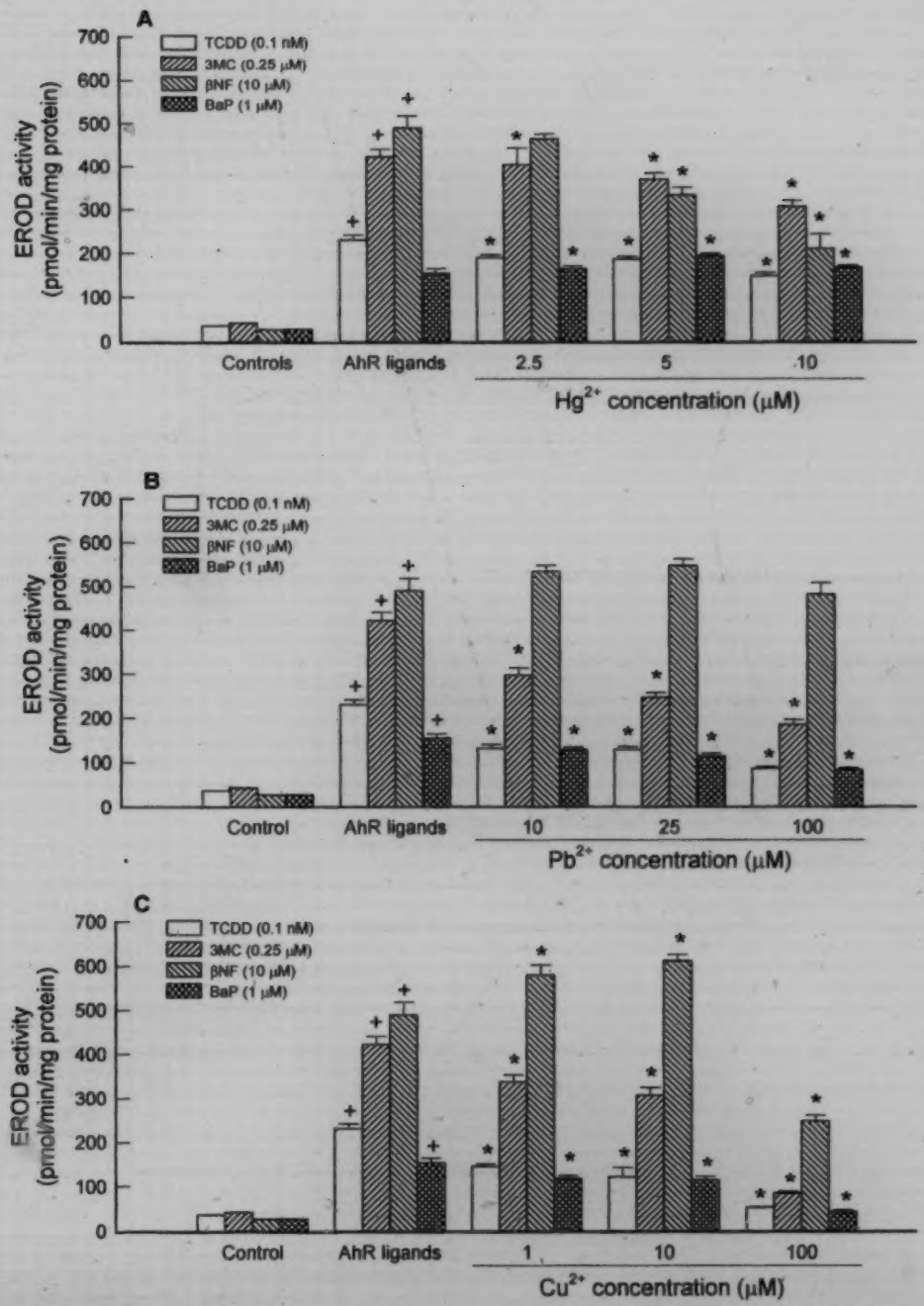

Figure 3.2. Effect of metals on the induction of EROD activity in WT cells. Hepa 1c1c7 cells were treated for 24 h with (A) Hg^{2+}, (B) Pb^{2+}, or (C) Cu^{2+} in the presence of AhR ligands, TCDD (0.1 nM), 3MC (0.25 μM), BaP (1 μM), or βNF (10 μM). EROD activity was measured in intact living cells using a 96-well plate fluorescent assay. Values are presented as mean ± SEM. (n=8). $^{+}P<0.05$ compared to control, $^{*}P<0.05$ compared to AhR ligands.

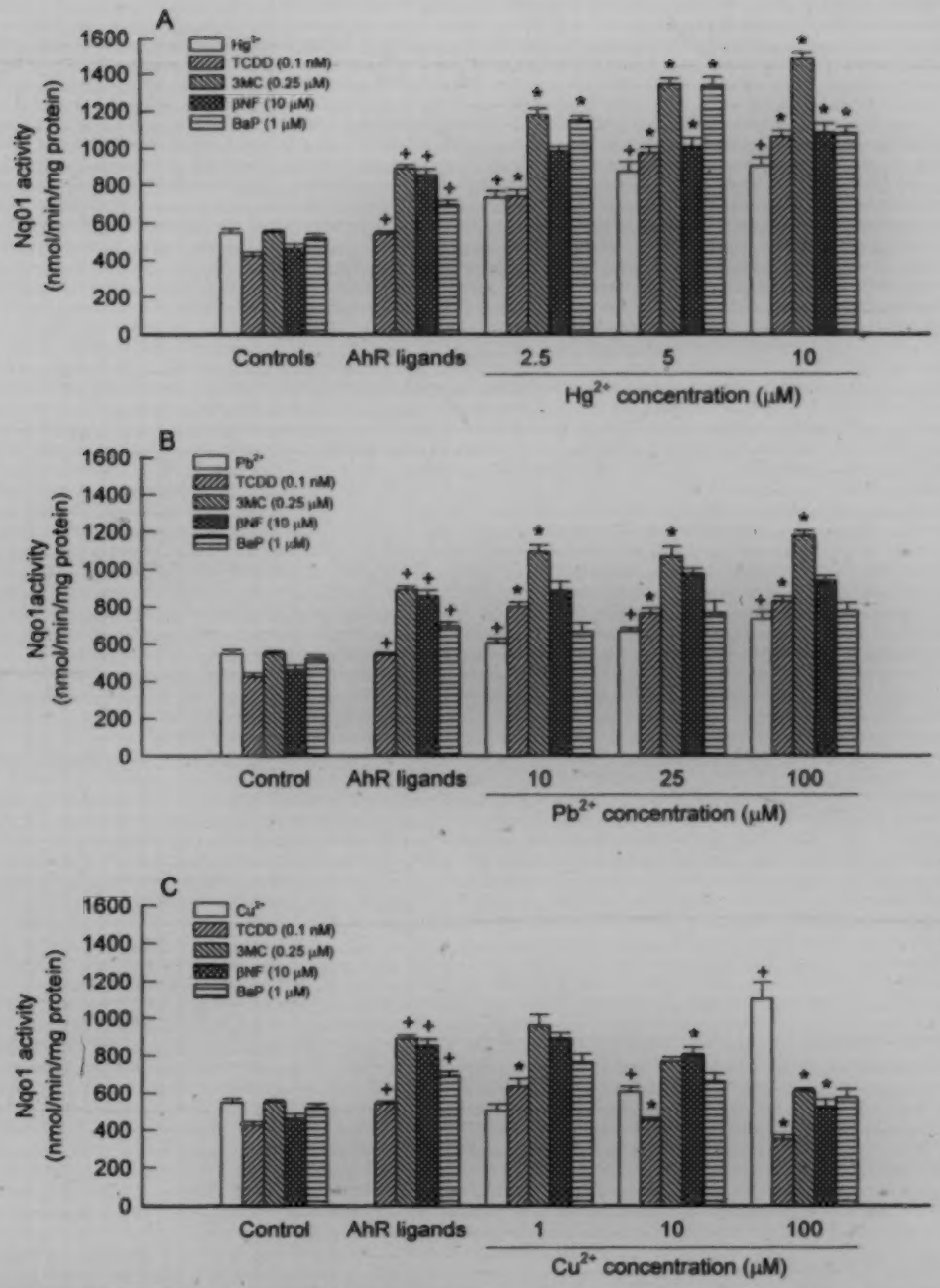

Figure 3.3. Effect of metals on the Nqo1 activity in WT cells. Cells were treated for 24 h with increasing concentrations of (A) Hg^{2+}, (B) Pb^{2+}, or (C) Cu^{2+}in the presence and absence of AhR ligand, TCDD, 3MC, βNF, or BaP. Nqo1 activity was determined spectrophotometricaly using DCPIP as a substrate. Values are presented as mean ± SEM. (n=8). $^{+}P<0.05$ compared to control, $^{*}P<0.05$ compared to AhR ligands

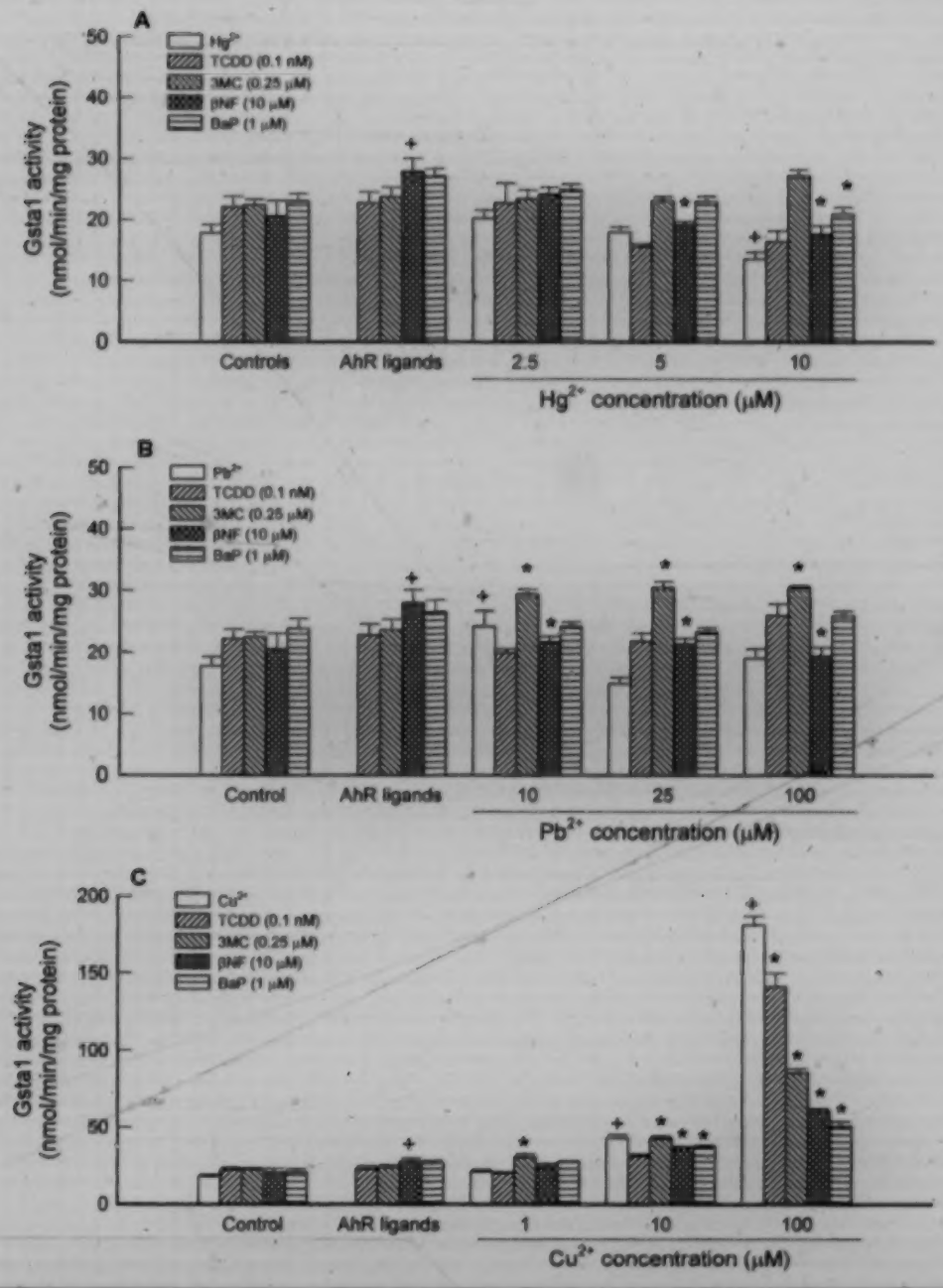

Figure 3.4. Effect of metals on the Gsta1 activity in WT cells. Cells were treated for 24 h with increasing concentrations of (A) Hg^{2+}, (B) Pb^{2+}, or (C) Cu^{2+} in the presence and absence of AhR ligand, TCDD, 3MC, βNF, or BaP. Gsta1 activity was determined spectrophotometricaly using CHP as substrate. Values are presented as mean ± SEM. (n=8). $^{+}P<0.05$ compared to control, $^{*}P<0.05$ compared to AhR ligands.

When metals were co-administered with AhR ligands, both Hg^{2+} and Pb^{2+} further potentiated, while Cu^{2+} decreased the induction of Nqo1 by AhR ligands in a dose-dependent manner (Fig. 3.3A–C). The maximum potentiation by Hg^{2+} and Pb^{2+} was observed with TCDD-induced Nqo1 activity, whereas the minimum induction was observed with BaP-induced Nqo1 activity (Fig. 3.3A and B). In contrast, Cu^{2+} showed the maximum inhibitory effect on βNF-induced Nqo1 activity (40%, $P<0.05$), as shown in Fig. 3.3C. With respect to Gsta1, Hg^{2+} significantly decreased βNF- and BaP-mediated effects on Gsta1 activity, while Cu^{2+} increased the activity by all AhR ligands in a concentration-dependent manner, as follows TCDD>3MC>βNF>BaP (Fig. 3.4A and C, respectively). Pb^{2+}, however, did not affect Gsta1 activity.

3.1.4. Effect of Metals on AhR-Regulated Genes in AhR-Deficient C12 Cells

To investigate whether the metal-mediated effects on the AhR-regulated genes at the activity levels are AhR-dependent, AhR-deficient C12 cells were exposed to a single concentration from each metal, Hg^{2+} (5 μM), Pb^{2+} (25 μM), and Cu^{2+} (10 μM), that did not significantly alter cell viability (Fig. 3.1B), in the absence and presence of AhR ligands. As expected, the Cyp1a1 activity was not detectable in C12 cells (data not shown). However, the specific basal activities of Nqo1 and Gsta1 in C12 cells were significantly decreased by 84% and 26%, respectively, compared to WT cells (Fig. 3.5). Interestingly, Hg^{2+} and Cu^{2+}, but not Pb^{2+}, significantly increased the Nqo1 and Gsta1 activities in C12 cells, respectively (Fig. 3.5). On the other hand, none of the AhR ligands tested induced Nqo1 and Gsta1 in C12 cells; however Hg^{2+} and Pb^{2+}, but not Cu^{2+}, significantly increased the induction of Nqo1 by all AhR ligands (Fig. 3.5A).

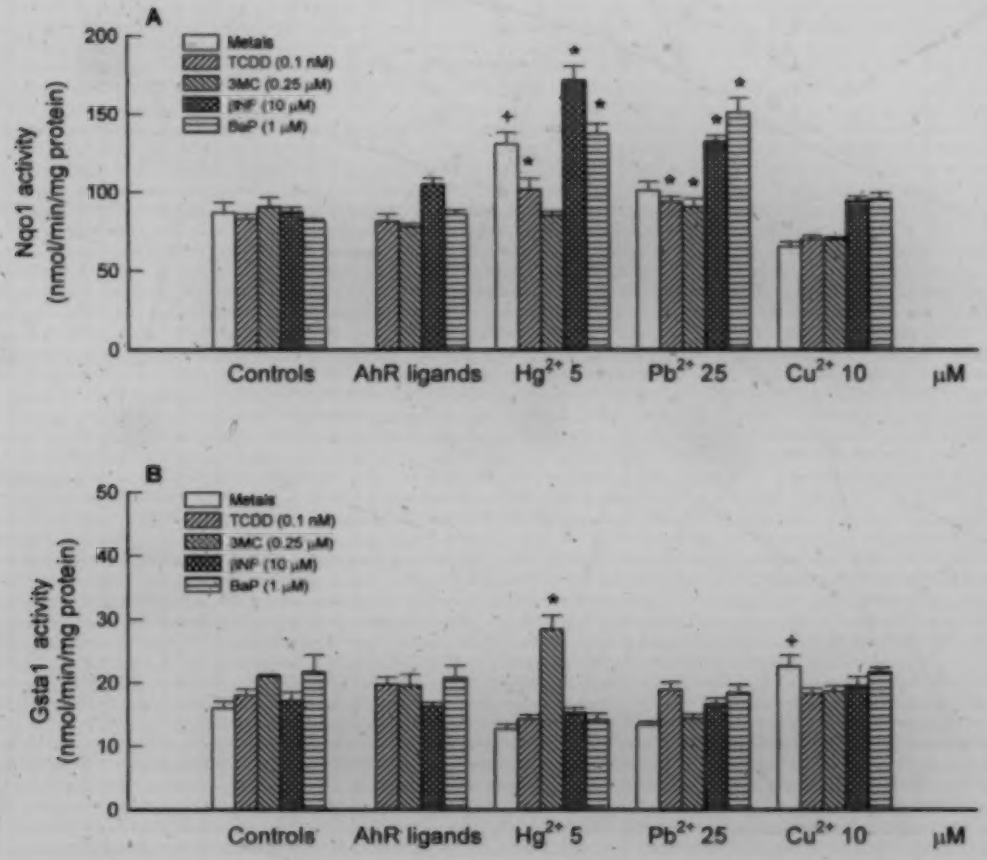

Figure 3.5. Effect of metals on the (A) Nqo1 and (B) Gsta1 activities in C12 cells. Cells were treated for 24 h with Hg^{2+} (5 μM), Pb^{2+} (25 μM), or Cu^{2+} (10 μM) in the presence and absence of AhR ligands, TCDD, 3MC, βNF, or BaP. Nqo1 and Gsta1 activities were determined spectrophotometricaly using DCPIP and CHP as substrates. Values are presented as mean ± SEM. (n=8). $^{+}P<0.05$ compared to control, $^{*}P<0.05$ compared to AhR ligands.

3.1.5. Effect of Metals on Constitutive and Inducible Cyp1a1 mRNA Level

To investigate whether metals alone are able to induce the expression of Cyp1a1 mRNA, WT cells were incubated with increasing concentrations of metals for 6 h. Interestingly, all three metals significantly induced Cyp1a1 mRNA levels in a concentration-dependent manner, with the only exception being that Cu^{2+} at the highest concentration did not induce Cyp1a1 expression (Fig. 3.6).

To determine if the metal-mediated inhibition of the AhR ligand-induced Cyp1a1 activity is associated with a decrease in the Cyp1a1 mRNA level, WT cells were exposed simultaneously to metals and AhR ligands. As expected, all AhR ligands significantly induced the Cyp1a1 mRNA expression, which is consistent with the activity results (Figs 3.7 – 3.10, lane 2). Interestingly, the inhibition of the AhR-inducible Cyp1a1 activity by Hg^{2+} was associated with an increase in the Cyp1a1 mRNA level (Figs. 3.7 – 3.10, lanes 3–5). On the other hand, Pb^{2+} did not significantly affect the 3MC-, βNF-, and BaP-mediated induction of Cyp1a1 mRNA but inhibited the TCDD-mediated Cyp1a1 mRNA induction (Figs. 3.7 – 3.10, lanes 6–8). In contrast, Cu^{2+} decreased the *Cyp1a1* gene expression by TCDD>βNF>3MC>BaP, which was in accordance with the activity results (Figs. 3.7 – 3.10, lanes 9–11). The discrepancies between metals in modulating the *Cyp1a1* gene expression suggest distinct transcriptional mechanisms may be involved.

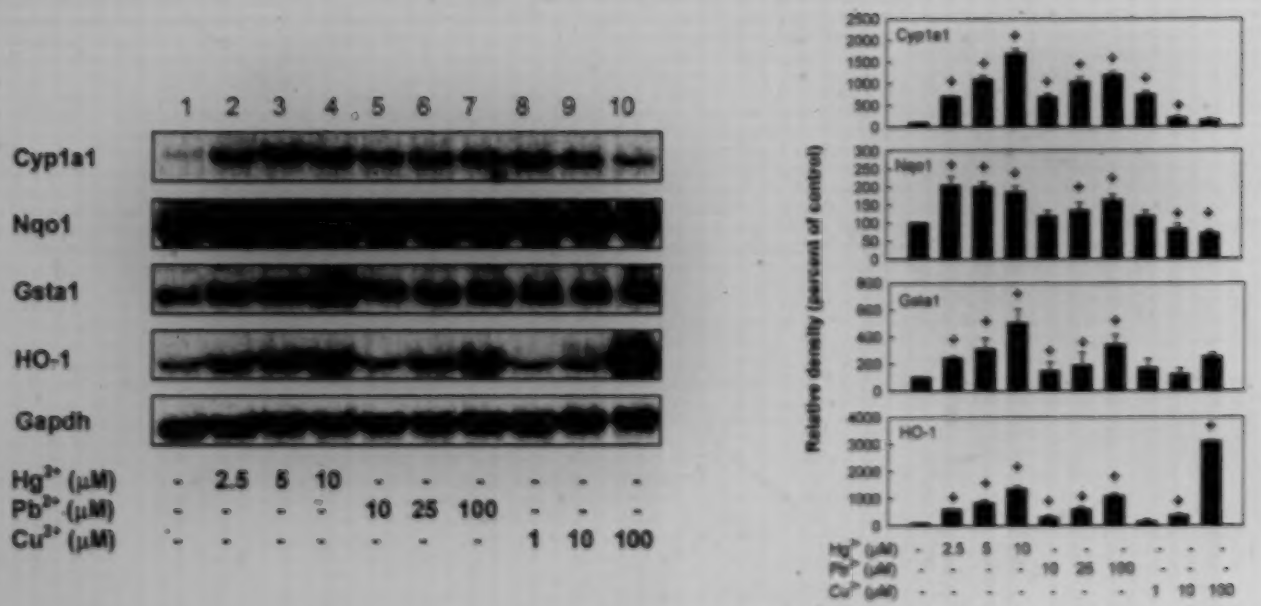
1 2 3 4 5 6 7 8 9 10
Cyp1a1
Nqo1
Gsta1
HO-1
Gapdh
Hg2+ (μM) - 2.5 5 10 - - - - - -
Pb2+ (μM) - - - - 10 25 100 - - -
Cu2+ (μM) - - - - - - - 1 10 100
Relative density (percent of control)
Cyp1a1
Nqo1
Gsta1
HO-1

Figure 3.6. Effect of metals on Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels in WT cells. Cells were treated for 6 h with increasing concentrations of Hg^{2+} (2.5, 5 and 10 μM), Pb^{2+} (10, 25 and 100 μM), or Cu^{2+} (1, 10 and 100 μM). Total RNA (20 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes, and hybridized with a [^{32}P]-labeled cDNA probe specific for mouse Cyp1a1. The blots were subsequently stripped and rehybridized sequentially with a cDNA probe specific for Nqo1, Gsta1, HO-1, and Gapdh. One of three representative experiments is shown. The graph represents the relative normalized amount of mRNA (mean ± SEM, n=3), which was calculated by dividing the levels Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels by the level of Gapdh mRNA in the same sample, and the results are expressed as percentage of the control values taken as 100%. $^{*}P<0.05$ compared to control (untreated cells).

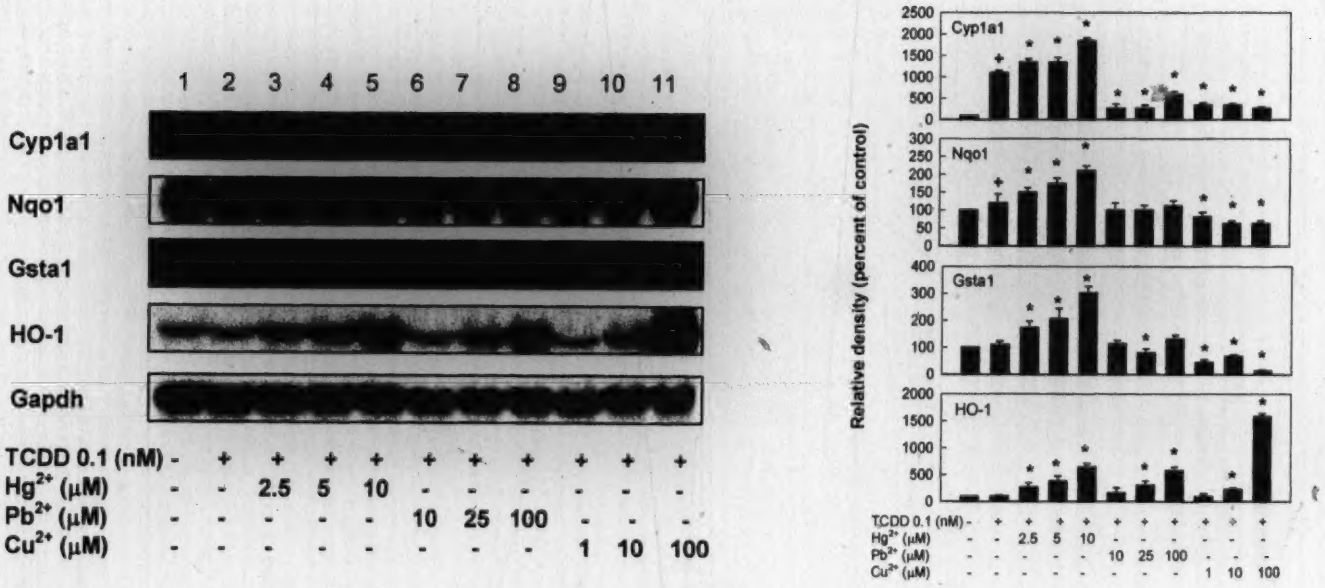
1 2 3 4 5 6 7 8 9 10 11
Cyp1a1
Nqo1
Gsta1
HO-1
Gapdh
TCDD 0.1 (nM) - + + + + + + + + + +
Hg2+ (μM) - - 2.5 5 10 - - - - - -
Pb2+ (μM) - - - - - 10 25 100 - - -
Cu2+ (μM) - - - - - - - - 1 10 100
Relative density (percent of control)
Cyp1a1
Nqo1
Gsta1
HO-1

Figure 3.7. Effect of co-administration of metals and TCDD on Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels in WT cells. Cells were treated for 6 h with increasing concentrations of Hg^{2+}, Pb^{2+}, or Cu^{2+} in the presence of TCDD. Cyp1a1, Nqo1, Gsta1, and HO-1 mRNAs normalized to Gapdh were determined by Northern blot analysis. One of three representative experiments is shown. The graph represents the relative normalized amount of mRNA (mean ± SEM, n=3), expressed as percentage of the control values taken as 100%. $^{+}P<0.05$ compared to control (untreated cells) and $^{*}P<0.05$ compared to TCDD.

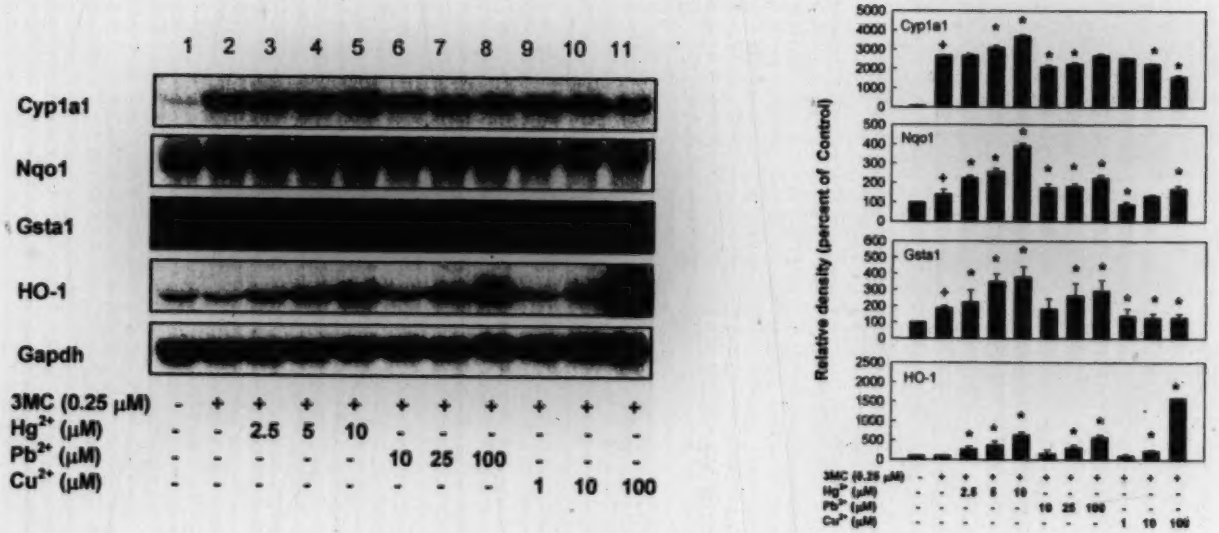
1 2 3 4 5 6 7 8 9 10 11
Cyp1a1
Nqo1
Gsta1
HO-1
Gapdh
3MC (0.25 μM) - + + + + + + + + + +
Hg2+ (μM) - - 2.5 5 10 - - - - - -
Pb2+ (μM) - - - - - 10 25 100 - - -
Cu2+ (μM) - - - - - - - - 1 10 100
Relative density (percent of Control)
Cyp1a1
Nqo1
Gsta1
HO-1

Figure 3.8. Effect of co-administration of metals and 3MC on Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels in WT cells. Cells were treated for 6 h with increasing concentrations of Hg^{2+}, Pb^{2+}, or Cu^{2+} in the presence of 3MC. Cyp1a1, Nqo1, Gsta1, and HO-1 mRNAs normalized to Gapdh were determined by Northern blot analysis. One of three representative experiments is shown. The graph represents the relative normalized amount of mRNA (mean ± SEM, n=3), expressed as percentage of the control values taken as 100%. $^{+}P<0.05$ compared to control (untreated cells) and $^{*}P<0.05$ compared to 3MC.

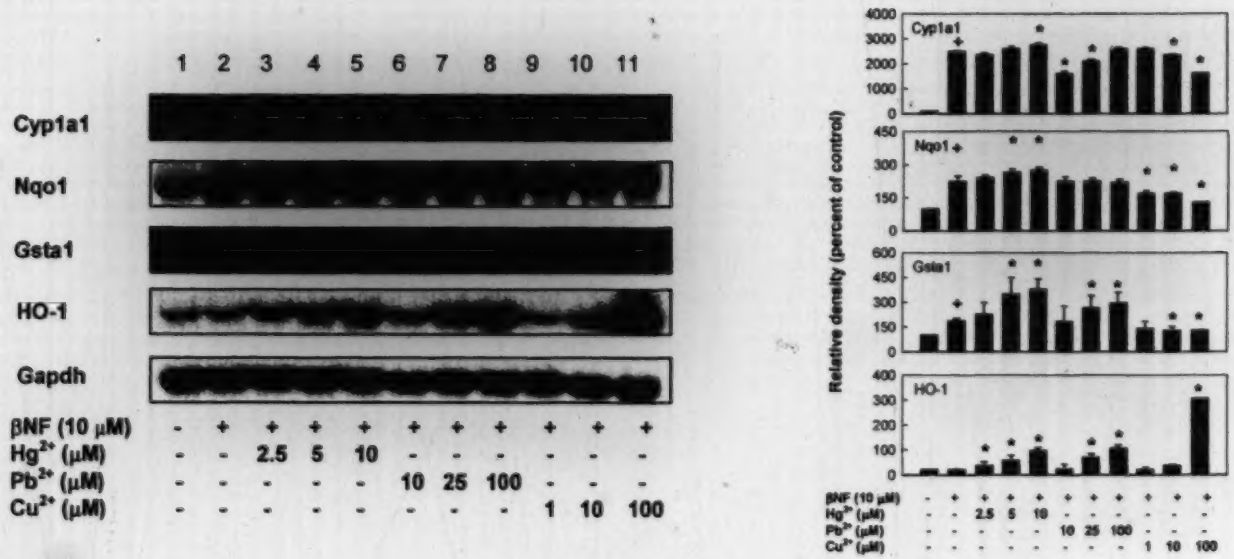
1 2 3 4 5 6 7 8 9 10 11
Cyp1a1
Nqo1
Gsta1
HO-1
Gapdh
βNF (10 μM) - + + + + + + + + + +
Hg2+ (μM) - - 2.5 5 10 - - - - - -
Pb2+ (μM) - - - - - 10 25 100 - - -
Cu2+ (μM) - - - - - - - - 1 10 100
Relative density (percent of control)
Cyp1a1
Nqo1
Gsta1
HO-1

Figure 3.9. Effect of co-administration of metals and βNF on Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels in WT cells. Cells were treated for 6 h with increasing concentrations of Hg^{2+}, Pb^{2+}, or Cu^{2+} in the presence of βNF. Cyp1a1, Nqo1, Gsta1, and HO-1 mRNAs normalized to Gapdh were determined by Northern blot analysis. One of three representative experiments is shown. The graph represents the relative normalized amount of mRNA (mean ± SEM, n=3), expressed as percentage of the control values taken as 100%. $^{+}P<0.05$ compared to control (untreated cells) and $^{*}P<0.05$ compared to βNF.

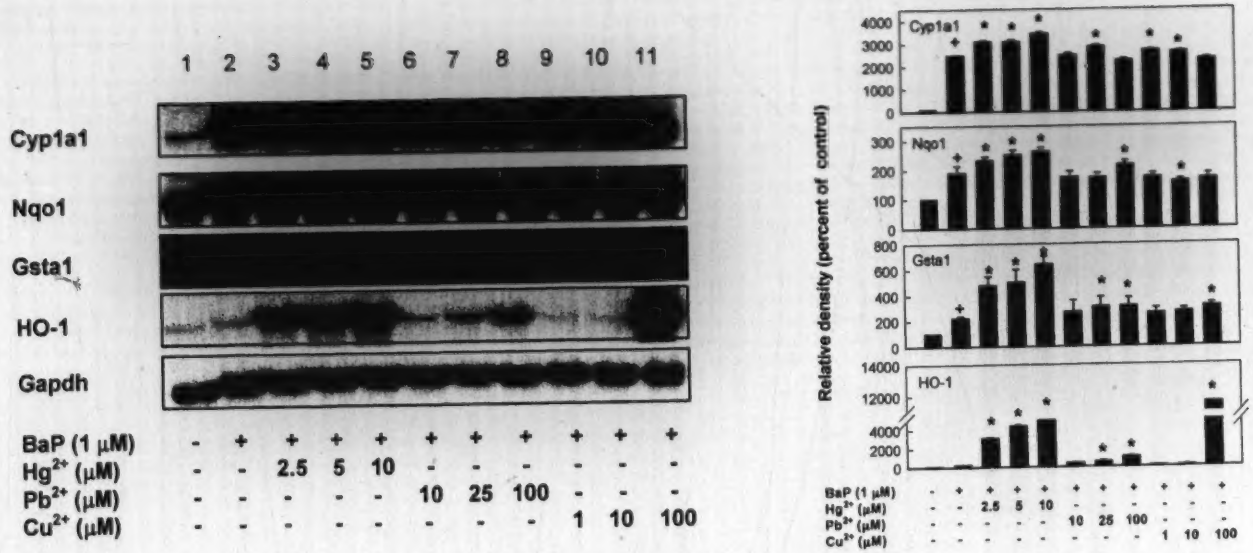

Figure 3.10. Effect of co-administration of metals and BaP on Cyp1a1, Nqo1, Gsta1, and HO-1 mRNA levels in WT cells. Cells were treated for 6 h with increasing concentrations of Hg^{2+}, Pb^{2+}, or Cu^{2+} in the presence of BaP. Cyp1a1, Nqo1, Gsta1, and HO-1 mRNAs normalized to Gapdh were determined by Northern blot analysis. One of three representative experiments is shown. The graph represents the relative normalized amount of mRNA (mean ± SEM, n=3), expressed as percentage of the control values taken as 100%. $^{+}P<0.05$ compared to control (untreated cells) and $^{*}P<0.05$ compared to BaP.

3.1.6. Effect of Metals on Constitutive and Inducible Nqo1 and Gsta1 mRNA Levels

To examine if the induction of Nqo1 and Gsta1 catalytic activities by AhR ligands is associated with the increase in mRNA expression, WT cells were treated with metals alone or with AhR ligands. Our results showed that all metals alone significantly induced Nqo1 and Gsta1 mRNA levels in a dose-dependent manner (Fig. 3.6). The potency of induction of both Nqo1 and Gsta1 by metals was in the following order Hg^{2+}>Pb^{2+}>Cu^{2+} (Fig. 3.6, lanes 4, 7 and 10). Thus, the Hg^{2+}-, Pb^{2+}-, and Cu^{2+}-mediated increase in Nqo1 activity coincided with the increase in gene transcription, suggesting that these metals induce Nqo1 at the transcription level. On the other hand, we have shown that all AhR ligands at the concentration used in this study induced the Nqo1 mRNA (βNF>BaP>3MC>TCDD), but failed to induce Gsta1, with the exception of βNF and BaP which induced Gsta1 mRNA (Figs. 3.9 and 3.10, lane 2, respectively).

When the WT cells were co-treated with metals and AhR ligands, our results showed that Hg^{2+} and Pb^{2+} increased, but Cu^{2+} deceased, the induction of Nqo1 and Gsta1 mRNA levels by all AhR ligands in a concentration-dependent manner (Fig. 3.7 – 3.10, lanes 3–11). The induction of Nqo1 and Gsta1 mRNA was maximally increased by Hg^{2+}. On the other hand, TCDD-mediated induction of Nqo1 and Gsta1 mRNA levels was decreased maximally with Cu^{2+} by about 48 and 30%, respectively, (Fig. 3.7, lanes 9–11).

3.1.7. Effect of Metals on HO-1 mRNA Level

To determine if the modulation of the AhR-regulated genes by metals is mediated, at least in part, by the oxidative stress protein HO-1, Northern blot analysis was carried out for HO-1 mRNA (Figs. 3.6 – 3.10). Our results clearly showed a significant and a marked increase in HO-1 mRNA level by all metals with or without AhR ligands in a concentration-dependent manner (Figs. 3.6-3.11). Treatment with AhR ligands alone did not significantly induce the mRNA expression of HO-1. Densitometric analysis for mRNA of HO-1 showed that the Cu^{2+} alone or with AhR ligands was clearly the most potent inducer of HO-1 followed by Hg^{2+} and Pb^{2+}. Furthermore, the highest induction of HO-1 mRNA by Hg^{2+} and Cu^{2+} was observed with BaP-treated WT cells (12- and 46-fold, respectively), whereas Pb^{2+} maximally induced HO-1 mRNA in TCDD-treated WT cells (six-fold). Interestingly, the increase in HO-1 gene expression coincided with the decrease in cyp1a1 and the increase in Nqo1 and Gsta1 catalytic activities, (Figs. 3.7 – 3.10).

3.2. MOLECULAR MECHANISMS INVOLVED IN THE MODULATION OF *Cyp1a1* GENE EXPRESSION BY Hg^{2+}, Pb^{2+}, AND Cu^{2+}

To explore the mechanisms by which metals modulate the expression of Cyp1a1 at the constitutive levels, we used single but different concentrations for each metal, which were 5, 25, and 10 μM for Hg^{2+}, Pb^{2+}, and Cu^{2+}, respectively. On the other hand, TCDD at a concentration of 1 nM was used to examine the effects of these metals at the inducible levels. It should be noted here that these concentrations of metals and TCDD metals used in the current study were chosen after determining their ability to cause submaximal effects and modulate *Cyp1a1* gene expression without significantly affecting Hepa 1c1c7 cell viability (Figs. 3.1 - 3.2 and 3.6 - 3.10).

3.2.1. Time-Dependent Effects of Metals on the Constitutive and Inducible Expression of Cyp1a1 mRNA

To better understand the kinetics of Cyp1a1 mRNA in response to metals, in comparison with TCDD, constitutive and TCDD-indueible Cyp1a1 mRNA contents were measured at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with vehicle, 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of 1 nM TCDD. Figure 3.11 shows that the Cyp1a1 mRNA was detectable as early as 3 h and remained elevated for at least 24 h after Hg^{2+}, Pb^{2+}, or Cu^{2+} treatment. The maximal induction (12-fold) was somewhat delayed and occurred at 12 h, followed by a 20% drop of the maximal level at 24 h. Interestingly, the kinetics of Cyp1a1 mRNA in the presence of metals was consistent with those obtained with TCDD, in which the onset of Cyp1a1

mRNA induction mediated by TCDD occurred at 3 h and reached the steady state (110-fold) at 6 h, followed by a 30% decline (Fig. 3.11). Vehicle alone did not significantly alter the Cyp1a1 mRNA at the various time points tested (data not shown). At the inducible level, cotreatment of the cells with TCDD and either Hg^{2+} (30%) or Pb^{2+} (22%) further increased the induction of Cyp1a1 mRNA, while TCDD and Cu^{2+} diminished Cyp1a1 induction by approximately 27% (Fig. 3.11). Taken together, the changes in the steady-state Cyp1a1 mRNA level by metals reflect an alteration in the rate of synthesis and/or degradation of Cyp1a1 mRNA.

3.2.2. Effect of Metals on the TCDD Concentration-Dependent Induction of Cyp1a1 mRNA

To further examine the effect of metals on the kinetics of Cyp1a1 mRNA induced by different concentrations of TCDD, Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence or absence of increasing concentrations of TCDD (0.1, 1, and 10 nM). Thereafter, Cyp1a1 mRNA was measured using Northern blot analysis. TCDD alone caused a concentration-dependent increase in Cyp1a1 mRNA levels (Fig. 3.12). However, at the highest concentration tested, 10 nM, the induction of Cyp1a1 mRNA was less than that obtained with 1 nM concentration. Hg^{2+} or Pb^{2+} further increased the Cyp1a1 mRNA mediated by TCDD at all concentrations of TCDD tested. Cu^{2+}, on the other hand, decreased the Cyp1a1 mRNA induction mediated by TCDD (Fig. 3.12).

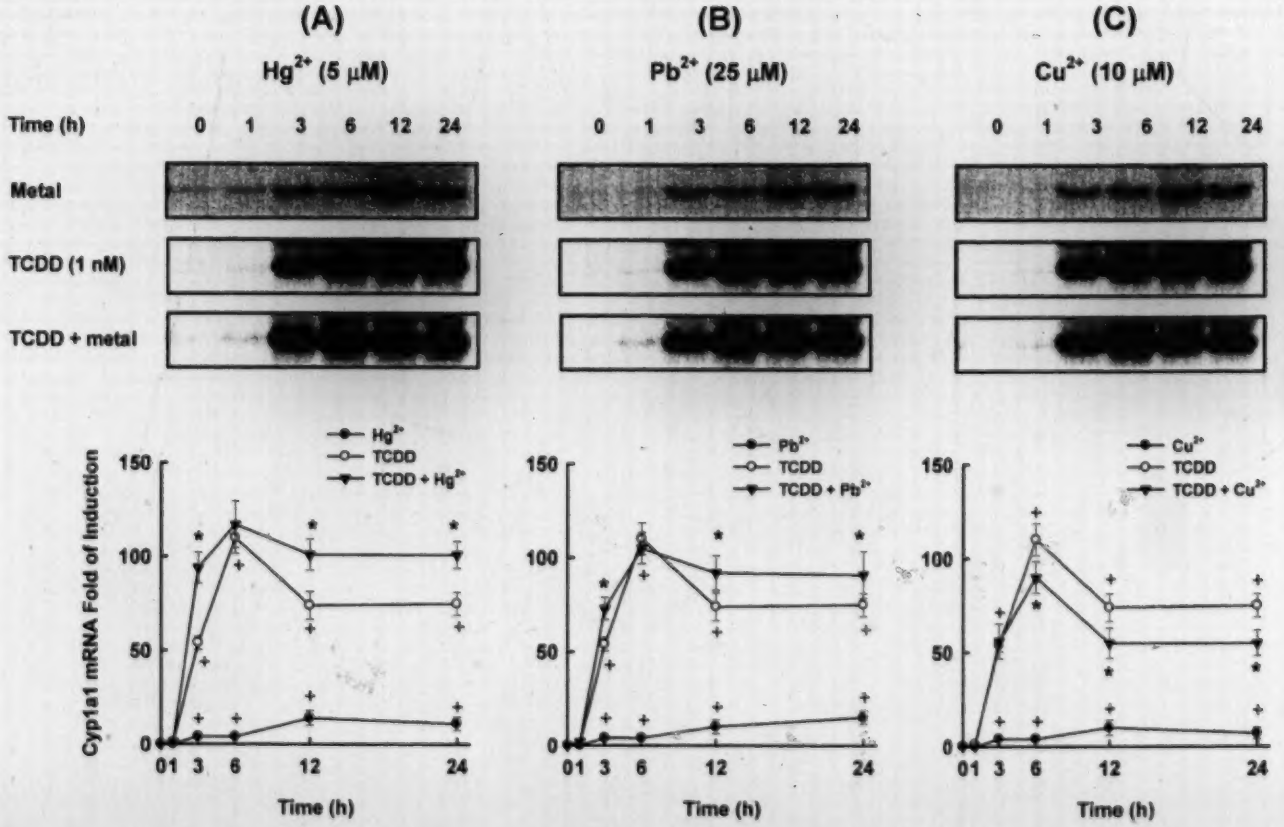

Figure 3.11. Time-dependent effects of metals on the constitutive and inducible expression of Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with (A) 5 μM Hg^{2+}, (B) 25 μM Pb^{2+}, or (C) 10 μM Cu^{2+} in the presence or absence of 1 nM TCDD for the time point indicated. Cyp1a1 mRNA levels normalized to Gapdh mRNA were detected by Northern blot analysis. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, n = 3), expressed as percent of the control (t=0). One of three representative experiments is shown. $^{+}p < 0.05$ compared with control, and $^{*}p < 0.05$ compared with TCDD.

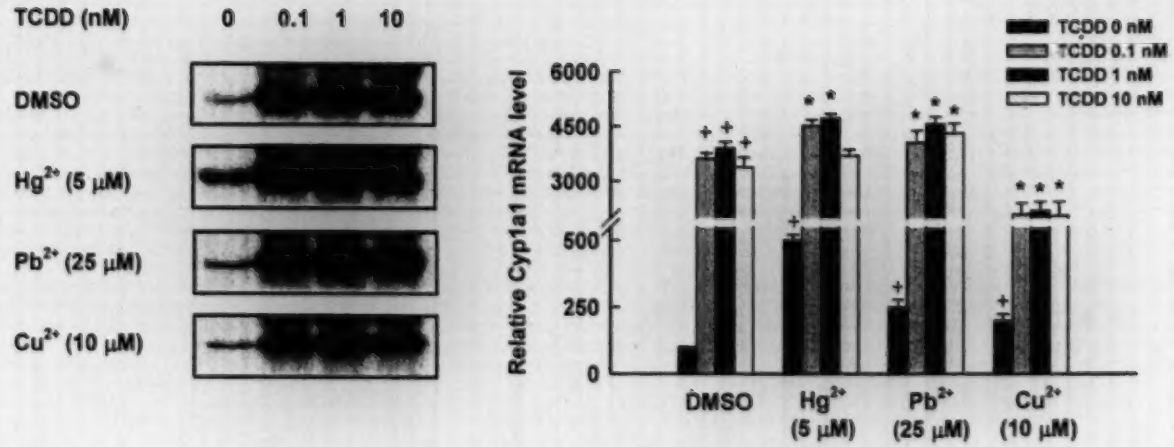

Figure 3.12. Effect of metals on the TCDD-concentration dependent effect on Cyp1a1 mRNA. Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence or absence of 0.1, 1, or 10 nM TCDD. Cyp1a1 mRNA levels normalized to Gapdh mRNA levels were determined by Northern blot analysis. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, $n = 3$), expressed as percent of the DMSO. One of three representative experiments is shown. $^{+}p < 0.05$ compared with DMSO, and $^{*}p < 0.05$ compared with corresponding TCDD in the absence of metal.

3.2.3. Transcriptional Regulation of *Cyp1a1* Gene by Metals

Initially, we questioned whether the induction of Cyp1a1 mRNA by metals (Figs. 3.11 and 3.12) is regulated at the transcriptional level. Therefore, a series of independent experiments were conducted.

3.2.3.1. Inhibition of Metal-Mediated Cyp1a1 mRNA Induction by an RNA Synthesis Inhibitor.

To investigate whether metals are able to increase the *de novo* Cyp1a1 RNA synthesis, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence or absence of 5 μg/ml Act-D, an RNA synthesis inhibitor. Total RNA was then isolated and quantified by Northern blot analysis. If metals increase the amount of Cyp1a1 mRNA through increasing its *de novo* RNA synthesis, we would expect to observe a decrease in the content of Cyp1a1 mRNA after the inhibition of its RNA synthesis.

Figure 3.13 shows that in untreated cells, as expected, the amount of Cyp1a1 mRNA was barely detectable (lane 1). However, pretreatment of the cells with Act-D for 6 h completely inhibited the constitutive expression of Cyp1a1 mRNA (lane 2). Metals alone significantly increased the Cyp1a1 mRNA level (lanes 3, 5, and 7). However, pretreatment of the cells with Act-D completely inhibited the metal-mediated Cyp1a1 mRNA induction (lanes 4, 6, and 8), in a manner similar to what was observed with TCDD (lane 9 and 10). These results suggest metals increase Cyp1a1 mRNA level by increasing its *de novo* RNA synthesis, in a manner similar to that observed with TCDD.

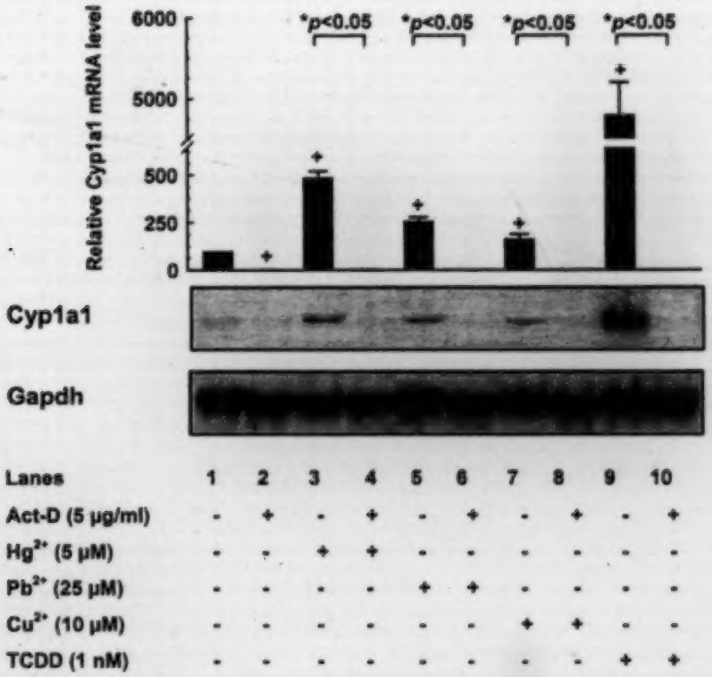
Relative Cyp1a1 mRNA level
6000
5000
500
250
0
*p<0.05
*p<0.05
*p<0.05
*p<0.05
Cyp1a1
Gapdh
Lanes 1 2 3 4 5 6 7 8 9 10
Act-D (5 µg/ml) - + - + - + - + - +
Hg2+ (5 µM) - - + + - - - - - -
Pb2+ (25 µM) - - - - + + - - - -
Cu2+ (10 µM) - - - - - - + + - -
TCDD (1 nM) - - - - - - - - + +

Figure 3.13. Effect of RNA synthesis inhibitor on metal-mediated increase in Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with 5 µg/ml Act-D, a RNA synthesis inhibitor, 30 min before exposure to 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 1 nM TCDD for an additional 6 h. Cyp1a1 mRNA normalized to Gapdh mRNA levels were detected by Northern blot analysis. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, $n = 3$), expressed as percent of the control. One of three representative experiments is shown. $^{+}p < 0.05$ compared with control, and $^{*}p < 0.05$ compared to same treatment in the absence of Act-D.

3.2.3.2. *Superinduction of Metal-Mediated Cyp1a1 mRNA Induction by an Protein Translation Inhibitor.*

To further confirm the transcriptional regulation of the *Cyp1a1* by metals, we examined the effect of a protein translation inhibitor, CHX. For this purpose, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence or absence of 10 μg/ml CHX, a concentration known to inhibit protein translation by 97% (Israel et al., 1985; Ma and Baldwin, 2002). Total RNA was then isolated and quantified by Northern blot analysis. If metals increase the transcription rate of Cyp1a1 under these conditions, we would expect to observe an increase in the accumulation of the Cyp1a1 mRNA after the inhibition of its protein translation.

As shown in Figure 3.14, CHX alone caused a significant accumulation of Cyp1a1 mRNA [6-fold greater than DMSO-treated cells (lane 2)]. However, cotreatment of the cells with CHX and metals further increased the accumulation of Cyp1a1 mRNA by CHX (lanes 4, 6, and 8), a phenomenon known as "superinduction" (Israel et al., 1985; Ma et al., 2000; Joiakim et al., 2004). Densitometric scanning of the autoradiogram indicates that the rates of Cyp1a1 mRNA synthesis induced by Hg^{2+}, Pb^{2+}, or Cu^{2+}, plus CHX were 2-, 3-, or 4-fold greater than those induced by each metal alone, respectively, and Cyp1a1 mRNA synthesis was about 25%, 37%, or 38% greater than that induced by CHX alone. Furthermore, the cotreatment of the cells with CHX and TCDD superinduced the increase in Cyp1a1 mRNA level by CHX (lane 10).

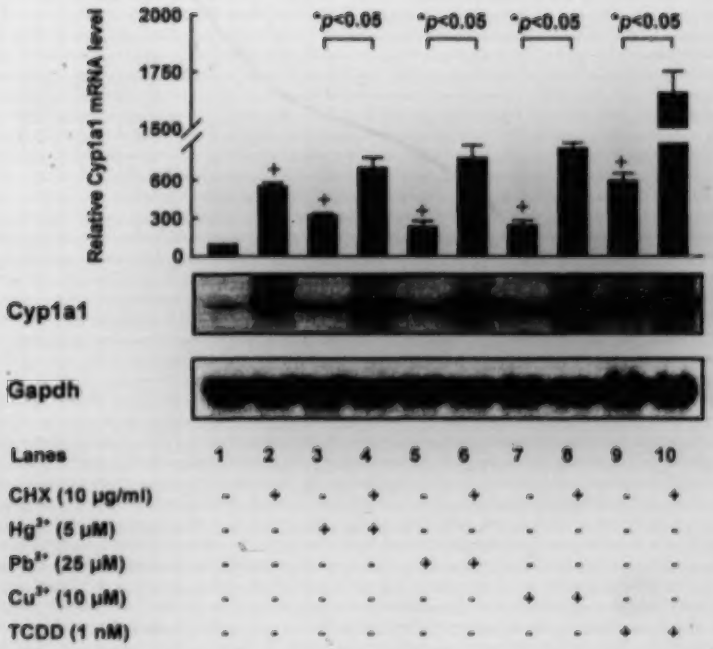
Relative Cyp1a1 mRNA level
2000
1750
1500
600
300
0
*p<0.05
*p<0.05
*p<0.05
*p<0.05
Cyp1a1
Gapdh
Lanes 1 2 3 4 5 6 7 8 9 10
CHX (10 µg/ml) - + - + - + - + - +
Hg2+ (5 µM) - - + + - - - - - -
Pb2+ (25 µM) - - - - + + - - - -
Cu2+ (10 µM) - - - - - - + + - -
TCDD (1 nM) - - - - - - - - + +

Figure 3.14. Effect of protein translation inhibitor on metal-mediated increase in Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with 10 µg/ml CHX, a protein translation inhibitor, 30 min before exposure to 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 1 nM TCDD for additional 6 h. Cyp1a1 mRNA normalized to Gapdh mRNA levels were detected by Northern blot analysis. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, n = 3), expressed as percent of the control. One of three representative experiments is shown. $^{+}p < 0.05$ compared with control, and $^{*}p < 0.05$ compared with same treatment in the absence of CHX

The inability of CHX to increase the mRNA level of Gapdh, a housekeeping gene used as a loading control, indicates that the superinduction by CHX is not a general effect. Thus, the superinduction of Cyp1a1 mRNA in response to metals by the protein translation inhibitor suggests the existence of a labile repressor protein required for Cyp1a1 transcription.

3.2.3.3. Superinduction of Metal-Mediated Cyp1a1 mRNA Induction by a 26S Proteasome Inhibitor.

Several studies have demonstrated that inhibition of proteasomal-dependent degradation of the AhR protein prolongs its half-life, thereby increasing the amount of functional AhR/ARNT complex and, hence, the transcription of Cyp1a1 in response to TCDD (Ma and Baldwin, 2002; Joiakim et al., 2004). The results of these studies suggest the involvement of proteasome-dependent pathway in the transcriptional regulation of Cyp1a1. To test the possibility that inhibition of proteasomal-dependent degradation of AhR protein would increase Cyp1a1 transcription in response to metals, Hepa 1c1c7 cells were pretreated with 25 μM MG-132, a potent 26S proteasome inhibitor (Ma et al., 2000), with or without 10 μg/ml CHX, both in the presence and absence of Hg^{2+}, Pb^{2+}, Cu^{2+}, or TCDD.

Treatment of Hepa 1c1c7 cells with either CHX or MG-132 alone for 6 h (Fig. 415), caused a 5- and 3-fold increase in the Cyp1a1 mRNA levels (lanes 2 and 3), respectively. Cotreatment of CHX and MG-132, however, did not further increase the accumulation of Cyp1a1 mRNA beyond what was observed with each inhibitor alone (lane 4). On the

other hand, treatment of the cells with CHX and metals or MG-132 and metals, superinduced Cyp1a1 mRNA expression. Contrastingly, when the cells were pretreated with CHX and MG-132 in the presence of metals, the superinduction of the Cyp1a1 mRNA was greater than the sum of the increases of Cyp1a1 mRNA that was observed with exposure to each inhibitor alone (lanes 6–8, 10–12, and 14–16). Similar results were observed when the cells were pretreated with CHX and/or MG-132 in the presence of TCDD (lanes 18–20). Taken together, these results suggest that the increased accumulation of Cyp1a1 mRNA in response to metals is an AhR-dependent mechanism.

3.2.3.4. Activation of AhR/XRE Binding by Metals.

The effect of metals on AhR translocation to the nucleus and the binding to the XRE of the *Cyp1a1* was measured by EMSA. Hepa 1c1c7 cells were treated with metals for 3 h, and their nuclear extracts were subjected to EMSA. Extracts from TCDD-treated cells were used as a positive control for AhR transformation. Figure 3.16A shows that Hg^{2+} or Pb^{2+}, but not Cu^{2+}, increased the DNA-binding capacity of the nuclear AhR, as shown by the intensity of the bands. The specificity of metal-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition assay in the presence of 100-fold molar excess of unlabeled XRE.

To further assess the ability of metals to directly activate AhR, EMSA was performed on untreated guinea pig hepatic cytosol previously incubated, *in vitro*, with either metals or TCDD for 3 h.

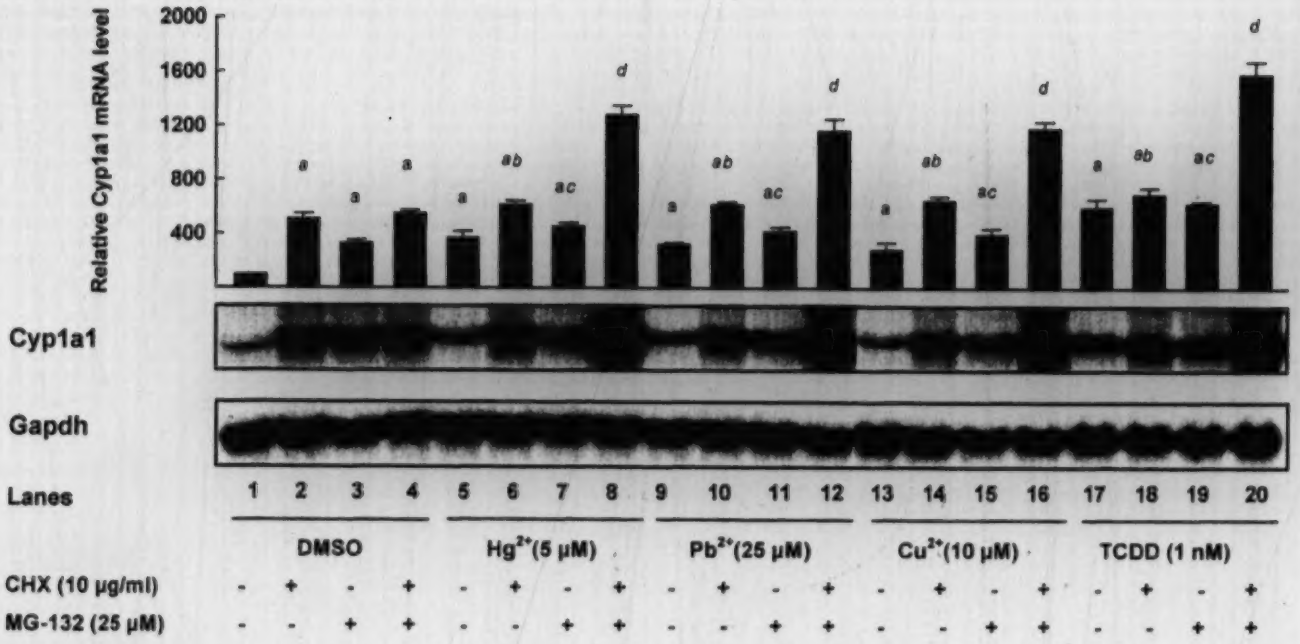

Figure 3.15. Effect of 26S proteasome inhibitor on metal-mediated increase in Cyp1a1 mRNA. Hepa 1c1c7 cells were treated with 10 μg/ml CHX, a protein synthesis inhibitor, 25 μM MG-132, 26S proteasome inhibitor, or CHX plus MG-132, 30 min before exposure to 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD for additional 6 h. Cyp1a1 mRNA normalized to Gapdh mRNA levels were detected by Northern blot analysis. The graph represents the relative amount of Cyp1a1 mRNA (mean ± SEM, $n = 3$), expressed as percent of the control. One of three representative experiments is shown.. $^{a}p < 0.05$ compared with DMSO, $^{b}p < 0.05$ compared with CHX, $^{c}p < 0.05$ compared with MG-132, and $^{d}p < 0.05$ compared with CHX plus MG-132 treatment.

Figure 3.16B shows that *in vitro* treatment of cytosol with metals, at the indicated concentrations, induced the transformation of the AhR/ARNT/XRE complex, as determined by the shifted bands, as compared to TCDD. This implies that metals are capable of directly activating the AhR. Taken together, the EMSA results provide strong evidence that the induction of Cyp1a1 mRNA by metals is both a transcriptional and an AhR-mediated event, in a manner similar to what was observed with TCDD.

3.2.4. Post-transcriptional Regulation of *Cyp1a1* by Metals

To further investigate if the observed increase in Cyp1a1 mRNA by metals could be attributed to a post-transcriptional stabilization of the mRNA, Act-D-chase experiments assessing the half-life of Cyp1a1 mRNA in the presence and absence of metals were performed. If metals do in fact stabilize the Cyp1a1 mRNA, an increase in mRNA half-life should be observed. As shown in Figure 3.17, Cyp1a1 mRNA decayed rapidly with an apparent half-life of 3.8 ± 0.33 h. In addition, all three metals did not significantly alter the half-life of Cyp1a1 mRNA, indicating that the increase of Cyp1a1 mRNA transcripts in response to metals was not due to a post-transcriptional stabilization of the mRNA.

3.2.5. Post-translational Regulation of *Cyp1a1* by Metals

The sustained increase in Cyp1a1 mRNA in response to metals (Figs. 3.11) prompted a further investigation into whether metals could modify the stability of Cyp1a1 protein. Therefore, the effect of metals on the Cyp1a1 protein half-life was determined using CHX-chase experiments. Figure 3.18 shows that Cyp1a1 protein is a short-lived protein with an estimated half-life of approximately 2.71 ± 0.02 h (Fig. 3.18). Interestingly, the

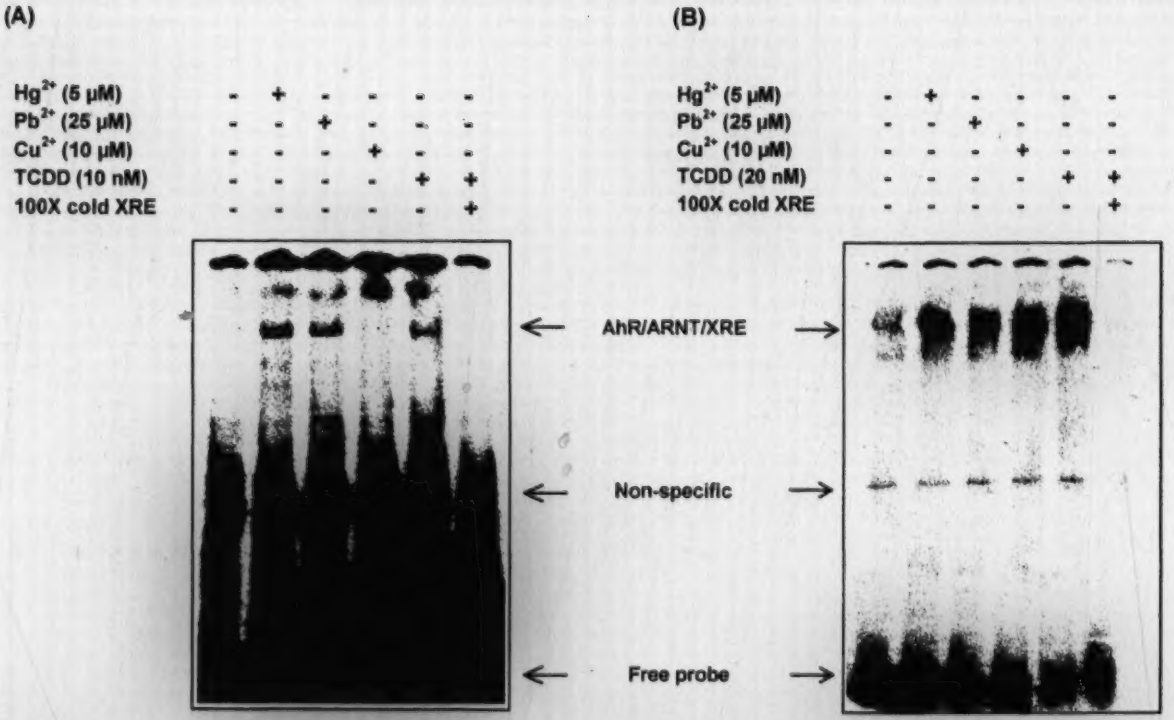
(A)
Hg2+ (5 µM) - + - - - -
Pb2+ (25 µM) - - + - - -
Cu2+ (10 µM) - - - + - -
TCDD (10 nM) - - - - + +
100X cold XRE - - - - - +
(B)
Hg2+ (5 µM) - + - - - -
Pb2+ (25 µM) - - + - - -
Cu2+ (10 µM) - - - + - -
TCDD (20 nM) - - - - + +
100X cold XRE - - - - - +
AhR/ARNT/XRE
Non-specific
Free probe

Figure 3.16. Metals induced AhR activation. (A) Nuclear extract (10 µg) from Hepa 1c1c7 cells treated with DMSO, 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 10 nM TCDD and (B) cytosolic extracts (80 µg) from untreated Guinea pig were incubated *in vitro* with DMSO, 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 20 nM TCDD, was mixed with ^{32}P-labelled XRE and the formation of AhR/ARNT/XRE complexes was analyzed by EMSA. The specificity of binding was determined by incubating the nuclear or cytosolic proteins treated with TCDD with 100-fold molar excess of unlabelled XRE. The arrows indicate the specific shift representing the AhR/ARNT/XRE complex. This pattern of AhR activation was observed in three separate experiments, and only one is shown.

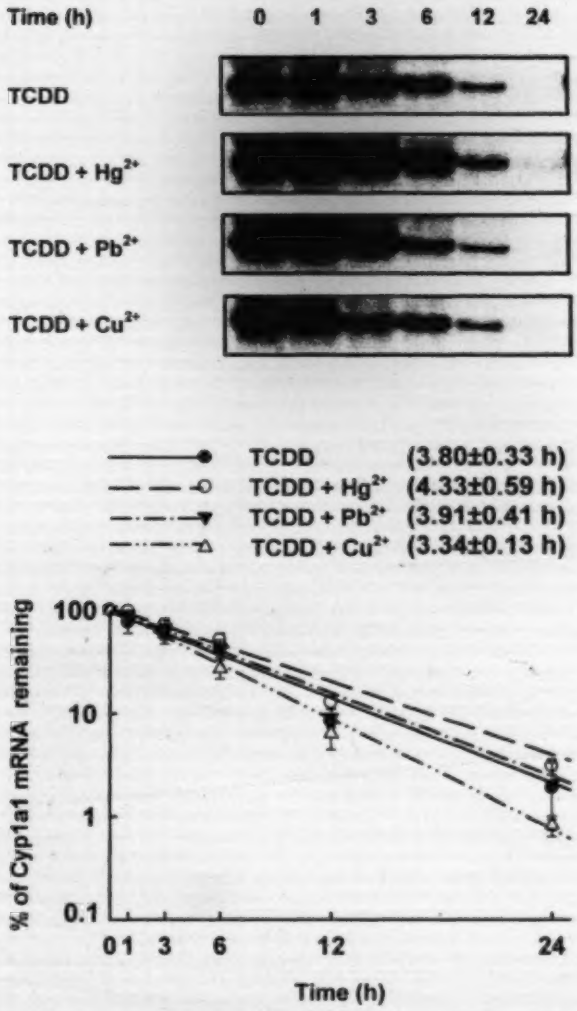

Figure 3.17. Effect of metals on the Cyp1a1 mRNA half-life. Hepa 1c1c7 cells were grown to 90% confluence in six-well cell culture plates and then treated with 1 nM TCDD for 12 h. The cells were then washed and incubated in a fresh media containing 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 5μg/ml Act-D, a RNA synthesis inhibitor. Cyp1a1 mRNA normalized to Gapdh was determined by Northern blot analysis. All mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of mRNA amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). One of three representative experiments is shown.

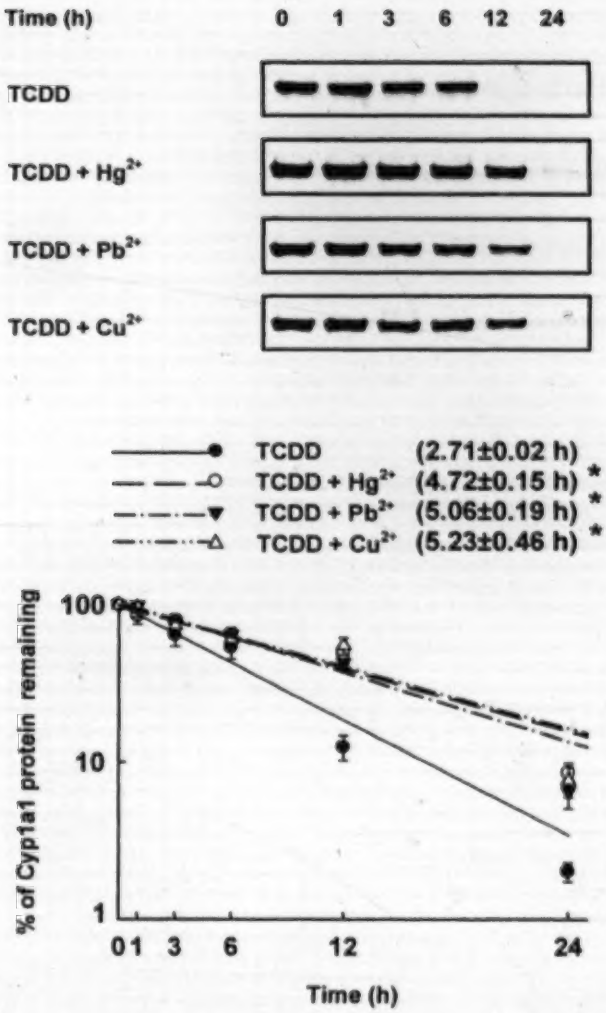

Figure 3.18. Effect of metals on the Cyp1a1 protein half-life. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then treated with 1 nM TCDD for 24 h. Thereafter, the cells were washed and incubated in fresh media containing 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 10 μg/ml CHX, a protein translation inhibitor. Cyp1a1 protein normalized to β-actin was determined by Western blot analysis. All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.85$) to a semilog plot of protein amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time, The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). $^*p < 0.05$ compared with TCDD

three metals significantly decreased the rate of Cyp1a1 protein degradation, implying a post-translational regulation of the Cyp1a1 by metals.

3.2.6. Inhibition of TCDD-Inducible Cyp1a1 Catalytic Activity by Metals is a Heme-Mediated Mechanism

To further examine whether the increases in Cyp1a1 mRNA and protein levels after metal treatments are reflected at the activity level, we investigated the effect of metals on the kinetics of the basal and TCDD-inducible Cyp1a1 activity. Therefore, Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of increasing concentrations of TCDD (0.1, 1, and 10 nM) for 24 h, followed by fluorimetric determination of Cyp1a1-mediated EROD catalytic activity.

TCDD alone caused a concentration-dependent increase in Cyp1a1 activity levels (Fig. 3.19A). However, at the highest concentration tested, 10 nM, the induction of Cyp1a1 activity was less than that obtained with 1 nM concentration (Fig. 3.19A). Although metals alone, in the absence of TCDD, did not significantly affect the Cyp1a1 activity, it was noted that the three metals significantly inhibited the induction of Cyp1a1 catalytic activity mediated by TCDD at concentrations 0.1 and 1 nM. However, at the highest concentration tested of TCDD, 10 nM, only Cu^{2+} decreased the Cyp1a1 catalytic activity (Fig. 3.19A).

The ability of metals to induce the Cyp1a1 mRNA, but not the catalytic activity, prompted further investigation. Therefore, we hypothesized that the decrease in TCDD-

inducible Cyp1a1 activity could be attributed to decreased cellular heme content. To test this hypothesis, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of increasing concentrations of TCDD (0.1, 1, and 10 nM) and Northern blot analysis for HO-1 mRNA, a rate limiting enzyme of heme degradation, was carried out. Our results clearly showed that all metals significantly increased HO-1 mRNA levels, which coincided with the inhibition of Cyp1a1 activity (Figs. 3.19A and B), indicating that all three metals increased heme degradation. To further confirm the role of heme degradation in reducing Cyp1a1 activity, we have measured the cellular heme content. Figure 3.19C shows that all three metals significantly decreased the cellular heme contents of TCDD-treated Hepa 1c1c7 cells.

Taken together, these results suggest that the decreased cellular heme content, at least in part, plays a role in reduced Cyp1a1 catalytic activity observed in metal-treated cells.

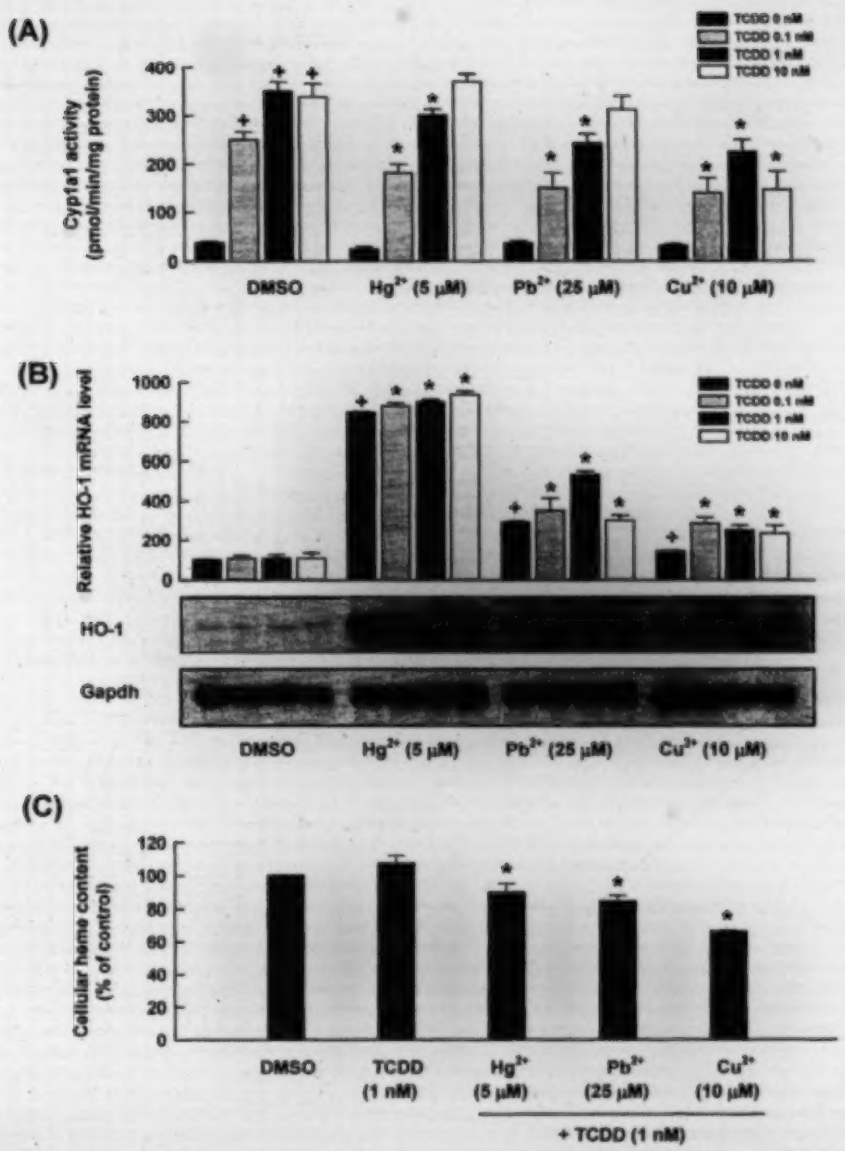

Figure 3.19. Effect of metals on the TCDD-concentration-dependent effect on (A) Cyp1a1 activity, (B) HO-1 mRNA, and (C) cellular heme content. Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of 0.1, 1, or 10 nM TCDD for indicated time. (A) Cyp1a1 activity was measured in intact living cells using a 96-well cell culture plates using 7ER as a substrate. Values are presented as mean ± SEM (n = 8). (B) HO-1 mRNA levels normalized to Gapdh were determined by Northern blot analysis. One of three representative experiments is shown. (C) Cellular heme content was measured by fluorimetric assay using excitation/emission wavelengths of 405/600 nm. Values are presented as mean ± SEM (n = 4). $^{+}p < 0.05$ compared to DMSO, and $^{*}p < 0.05$ compared to TCDD.

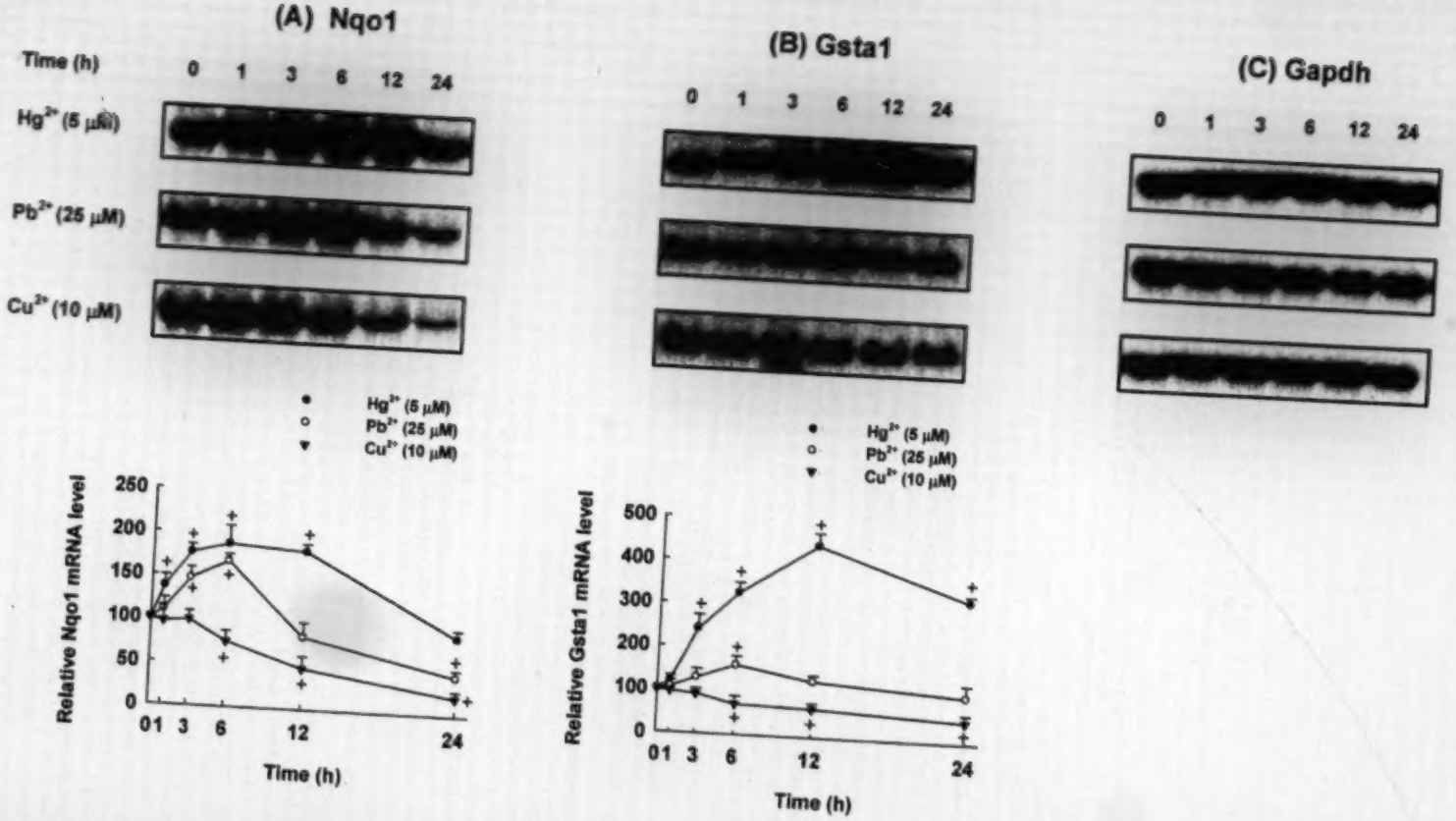

Figure 3.20. Time-dependent effects of metals on the constitutive expression of (A) Nqo1 and (B) Gsta1 mRNAs. Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} for the time point indicated. Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± SEM., $n = 3$), expressed as percent of the control (t=0) values taken as 100%. One of three representative experiments is shown. +, $p < 0.05$ compared with control.

3.3. MOLECULAR MECHANISMS INVOLVED IN THE MODULATION OF *Nqo1* AND *Gsta1* GENES EXPRESSION BY Hg^{2+}, Pb^{2+}, AND Cu^{2+}

3.3.1. Time-Dependent Effects of Metals on Constitutive and TCDD-Inducible Expression of Nqo1 and Gsta1 mRNAs.

To better understand the effect of metals on the kinetics of Nqo1 and Gsta1 mRNAs compared with TCDD, the constitutive and inducible expression of Nqo1 and Gsta1 mRNAs were measured at various time points (0, 1, 3, 6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the absence (Fig. 3.20A and B) and presence (Fig. 3.21A and B) of 1 nM TCDD.

At the constitutive level, Fig. 3.20A and B shows that Hg^{2+} and Pb^{2+} significantly increased the Nqo1 and Gsta1 mRNA transcripts in a time-dependent manner. Hg^{2+} treatment caused a maximal induction of Nqo1 (70%) and Gsta1 (250%) between 6 and 12 h. With Pb^{2+}, a 1.5-fold induction of Nqo1 and Gsta1 mRNAs occurred earlier, at 6 h, followed by a 70 (Nqo1) and 40% (Gsta1) drop of the maximal mRNA levels at 24 h. In contrast, Cu^{2+} caused a time-dependent decrease in the Nqo1 and Gsta1 mRNA levels (Fig. 3.20A and B).

At the inducible level, cotreatment of the cells with TCDD and either Hg^{2+} or Pb^{2+} further increased the TCDD-mediated induction of Nqo1 and Gsta1 mRNAs in a time-dependent manner, whereas cotreatment with TCDD and Cu^{2+} inhibited the induction (Fig. 3.21A and B). A densitometric scan of the autoradiogram indicates that Hg^{2+}, in the presence of

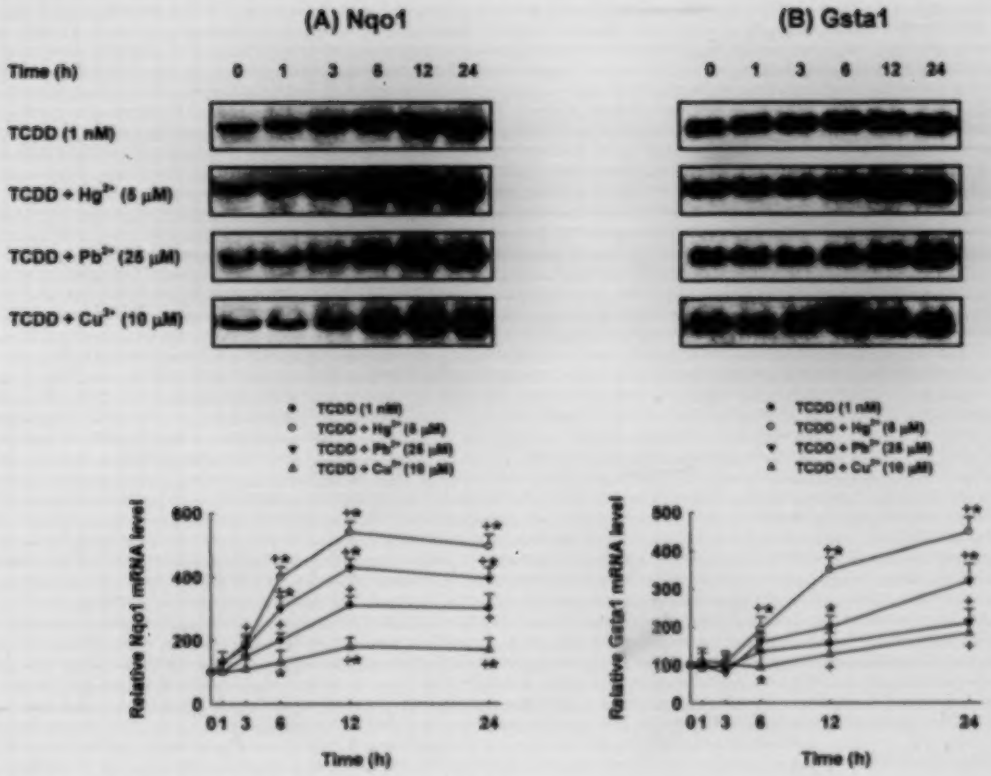

Figure 3.21. Time-dependent effects of metals on the inducible expression of (A) Nqo1 and (B) Gsta1 mRNAs. Hepa 1c1c7 cells were treated with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence of 1 nM TCDD for the time point indicated. Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± S.E.M., $n = 3$), expressed as percent of the control (t=0) values taken as 100%. One of three representative experiments is shown. +, $p < 0.05$ compared with control and *$p < 0.05$ compared to TCDD treatment.

TCDD, significantly increased the steady-state Nqo1 and Gsta1 mRNA levels by 1- and 2-fold, respectively. Yet, in the presence of Cu^{2+}, the steady-state mRNA levels of Nqo1 and Gsta1 were decreased by approximately 50 and 25%, respectively. Taken together, the changes in the steady state of Nqo1 and Gsta1 mRNA levels by metals reflect an alteration in the rate of synthesis and/or degradation of these genes.

3.3.2. Effects of Metals on the TCDD Concentration-Dependent Induction of Nqo1 and Gsta1 mRNAs and Activities.

To further examine the effect of metals on the kinetics of Nqo1 and Gsta1 mRNAs and activities induced by different concentrations of TCDD, Hepa 1c1c7 cells were treated with 5 μM Hg^{2+},25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of increasing concentrations of TCDD (0.1, 1, and 10 nM). Nqo1 and Gsta1 mRNAs and catalytic activities were measured using Northern blot and spectrophotometric assays, respectively.

At the mRNA level, TCDD treatment alone caused a concentration-dependent increase in Nqo1 and Gsta1 mRNA levels (Fig. 3.22A and B). Hg^{2+} and Pb^{2+} further increased the TCDD-mediated induction of Nqo1 and Gsta1 mRNAs at all concentrations of TCDD tested. Cu^{2+}, on the other hand, decreased the TCDD-mediated induction of both Nqo1 and Gsta1 mRNAs (Fig. 3.22A and B).

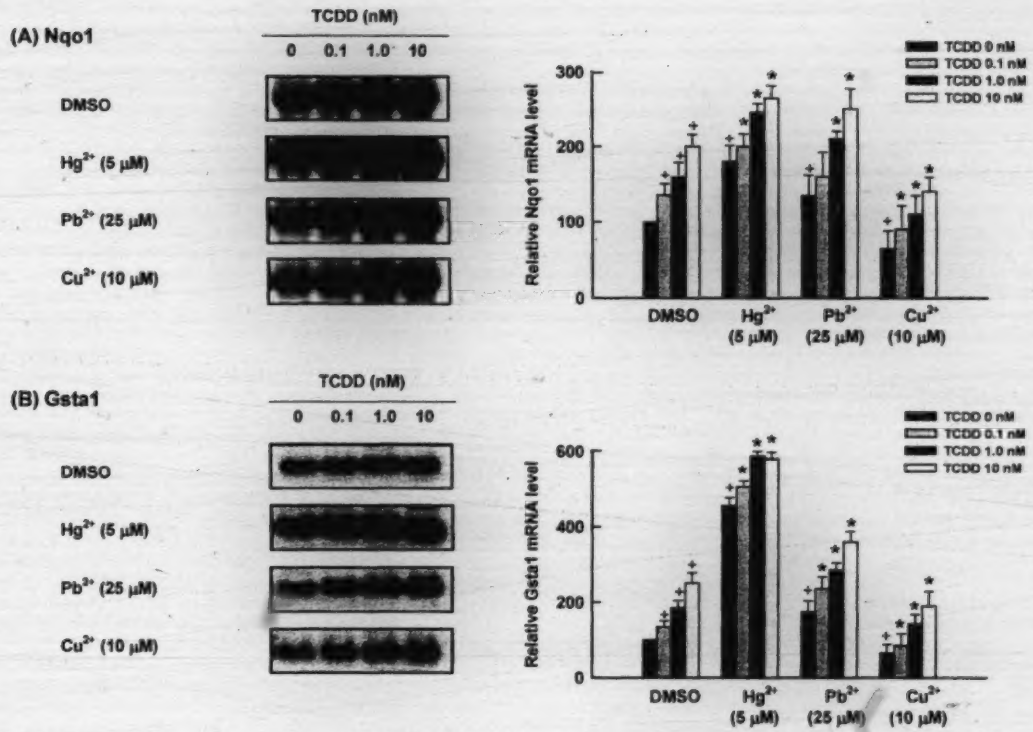

Figure 3.22. Effect of metals on the TCDD concentration-dependent induction of (A) Nqo1 and (B) Gsta1 mRNAs. Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of increasing concentrations of TCDD (0.1, 1, or 10 nM). Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± SEM., n = 3), expressed as percent of the control (concentration=0) values taken as 100%. One of three representative experiments is shown. +, $p < 0.05$ compared with control; and *, $p < 0.05$ compared with TCDD.

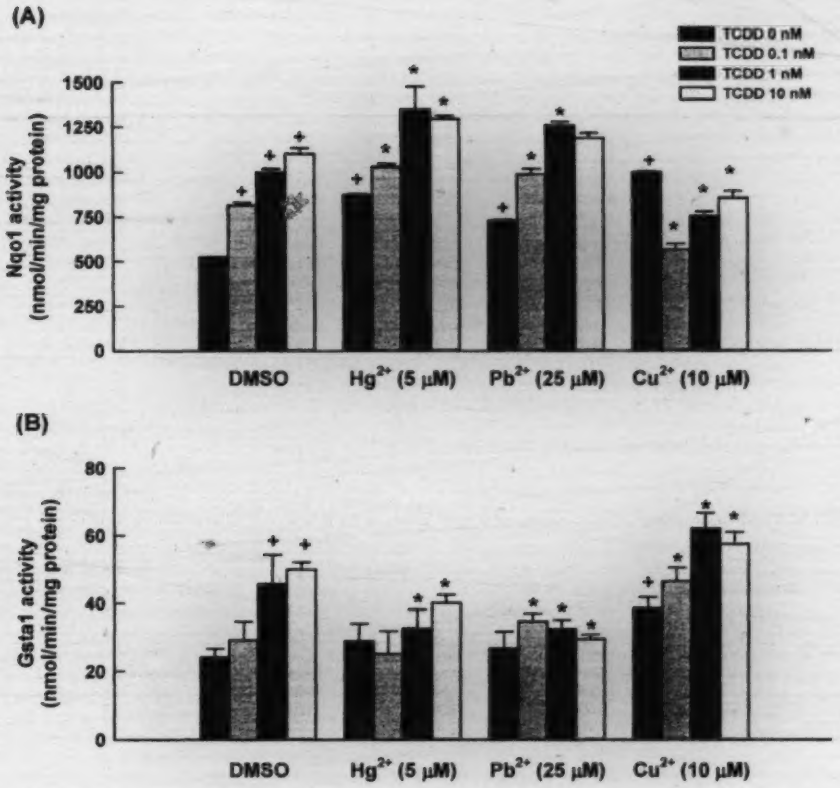

Figure 3.23. Effect of metals on the TCDD concentration-dependent induction of (A) Nqo1 and (B) Gsta1 activities. Hepa 1c1c7 cells were treated for 24 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} in the presence and absence of increasing concentrations of TCDD (0.1, 1, or 10 nM). Nqo1 and Gst ya enzyme activities were determined spectrophotometrically using DCPIP and CHP as substrates, respectively. Values are presented as mean ± SEM. (n = 6). +, $p < 0.05$ compared with control; and *, $p < 0.05$ compared with TCDD

At the activity levels, TCDD treatment alone induced the Nqo1 and Gsta1 catalytic activities in a concentration-dependent manner (Fig. 3.23A and B). At the constitutive level, all three metals significantly increased the Nqo1 activity (Fig. 3.23A); however, only Cu^{2+} significantly increased Gsta1 activity (Fig. 3.23B). At the inducible level, Hg^{2+} and Pb^{2+} further potentiated the TCDD-mediated induction of Nqo1 activity (Fig. 3.23A) but inhibited the induction of Gsta1 activity at all TCDD concentrations (Fig. 3.23B). In contrast, Cu^{2+} inhibited the induction of Nqo1 activity at all TCDD concentrations while further potentiating the TCDD-inducible Gsta1 activity (Fig. 3.23A and B).

3.3.3. Inhibition of Metal-Mediated Induction of Nqo1 and Gsta1 mRNAs by a RNA Synthesis Inhibitor.

To investigate whether metals are capable of increasing the *de novo* Nqo1 or Gsta1 RNA synthesis, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence and absence of 5 μg/ml Act-D, a RNA synthesis inhibitor. Total RNA was then isolated and quantified by Northern blot analysis. If metals increase the amount of Nqo1 or Gsta1 mRNA through increasing their *de novo* RNA synthesis, under these circumstances we would expect to observe a decrease in the content of Nqo1 or Gsta1 mRNA after the inhibition of their RNA synthesis.

Figure 3.24A and B, shows that in untreated cells, the Nqo1 and Gsta1 mRNAs are constitutively expressed (lane 1). However, pretreatment of the cells with Act-D for 6 h significantly inhibited the constitutive Nqo1 and Gsta1 mRNAs by approximately 23 and

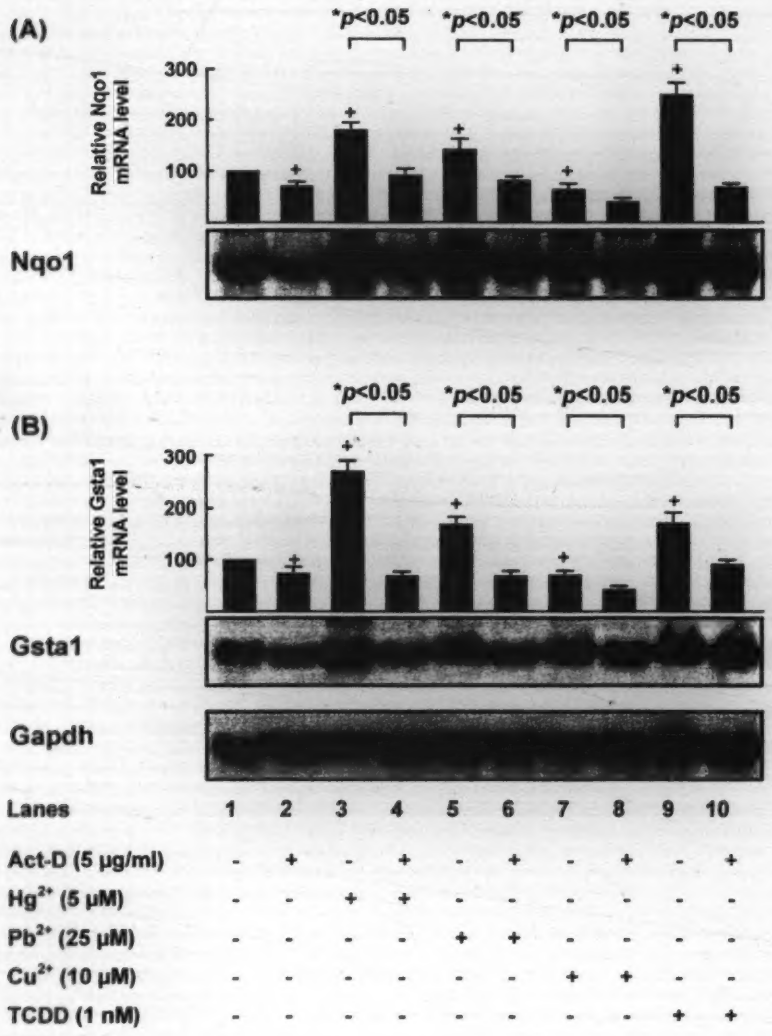

Figure 3.24. Effect of an RNA synthesis inhibitor on metal-mediated induction of (A) Nqo1 and (B) Gsta1 mRNAs. Hepa 1c1c7 cells were treated with 5 µg/ml Act-D, a RNA synthesis inhibitor, 30 min before exposure to 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 1 nM TCDD for additional 6 h. Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± S.E.M., $n = 3$), expressed as percent of the control. One of three representative experiments is shown. +, $p < 0.05$ compared with control and *, $p < 0.05$ compared with the same treatment in the absence of Act-D.

27%, respectively (lane 2). In metal-treated cells, Hg^{2+} and Pb^{2+} alone significantly induced Nqo1 mRNA by approximately 85 and 40% and Gsta1 mRNA by 170 and 70%, respectively (lanes 3 and 5). Cu^{2+}, on the other hand, inhibited the constitutive expression of both genes (lane 7). Treatment of the cells with Act-D completely inhibited the metal-mediated induction of Nqo1 and Gsta1 mRNAs (lanes 4, 6, and 8). Similar results were observed with TCDD in which the induction of Nqo1 (2-fold) and Gsta1 (1-fold) mRNAs was completely inhibited by Act-D (lanes 9 and 10). These results suggest that metals increased the Nqo1 and Gsta1 mRNA levels by increasing their *de novo* RNA synthesis in a manner similar to what was observed with TCDD.

3.3.4. Induction of Nqo1 and Gsta1 mRNAs in Response to Metals Requires a Labile Protein.

Several studies have shown that Nrf2, a labile transcriptional protein, is required for the induction of Nqo1 and Gsta1 genes by the XRE and ARE inducers (Ma et al., 2004; McWalter et al., 2004). To examine whether the effect of metals on Nqo1 and Gsta1 mRNAs requires *de novo* protein synthesis, we examined the effect of the protein synthesis inhibitor CHX at a concentration that has been shown to inhibit Nrf2 protein synthesis in Hepa 1c1c7 cells (Stewart et al., 2003; Ma et al., 2004). For this purpose, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence and absence of 10 μg/ml CHX. Total RNA was then isolated and quantified by Northern blot analysis. If the induction of Nqo1 and Gsta1 mRNAs in response to metals requires the existence of Nrf2, we would expect to observe a decrease

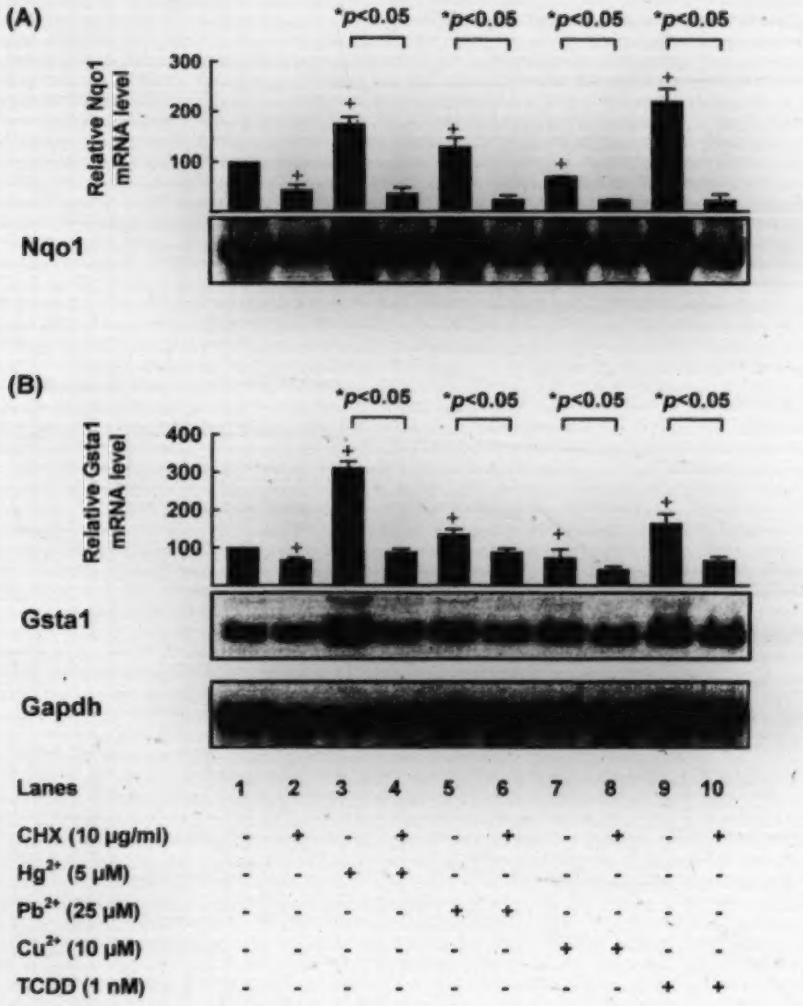

Figure 3.25. Effect of a protein synthesis inhibitor on metal-mediated induction of (A) Nqo1 and (B) Gsta1 mRNAs. Hepa 1c1c7 cells were treated with 10 µg/ml CHX, a protein synthesis inhibitor, 30 min before exposure to 5 µM Hg^{2+}, 25 µM Pb^{2+}, 10 µM Cu^{2+}, or 1 nM TCDD for 6 h. Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± SEM., $n = 3$), expressed as percent of the control. One of three representative experiments is shown. +, $p < 0.05$ compared with control and *, $p < 0.05$ compared with the same treatment in the absence of CHX.

in the mRNA levels after inhibiting protein synthesis since Nrf2 protein half-life is less than 30 min (Stewart et al., 2003; Ma et al., 2004).

As shown in Fig. 3.25, CHX alone caused a significant decrease in Nqo1 and Gsta1 mRNA levels by approximately 65 and 25%, respectively (lane 2). The difference in magnitude of inhibition of the constitutive Nqo1 or Gsta1 mRNA by CHX suggests that these genes are differentially regulated by the transcriptional protein. However, cotreatment of the cells with CHX significantly inhibited the metal-mediated induction of Nqo1 and Gsta1 mRNAs to the level of CHX alone (lanes 4, 6, and 8). Similarly, the TCDD-mediated induction of Nqo1 and Gsta1 mRNAs was completely blocked by CHX (lanes 9 and 10). These results suggest that the induction of Nqo1 and Gsta1 mRNAs by metals requires *de novo* CHX-sensitive labile protein synthesis in a manner similar to what was observed with TCDD.

3.3.5. Inhibition of Metal-Mediated Induction of Nqo1 and Gsta1 mRNAs by CHX is a Transcriptional Mechanism.

Inhibition of the metal-mediated induction of Nqo1 and Gsta1 mRNAs by CHX can be transcriptional, in which CHX inhibits RNA synthesis, or post-transcriptional, due to an increase in degradation rate. To distinguish between these possibilities, Hepa 1c1c7 cells were treated with either metals alone for 2 or 6 h, or with metals for 2 h followed by metals plus CHX for an additional 4 h.

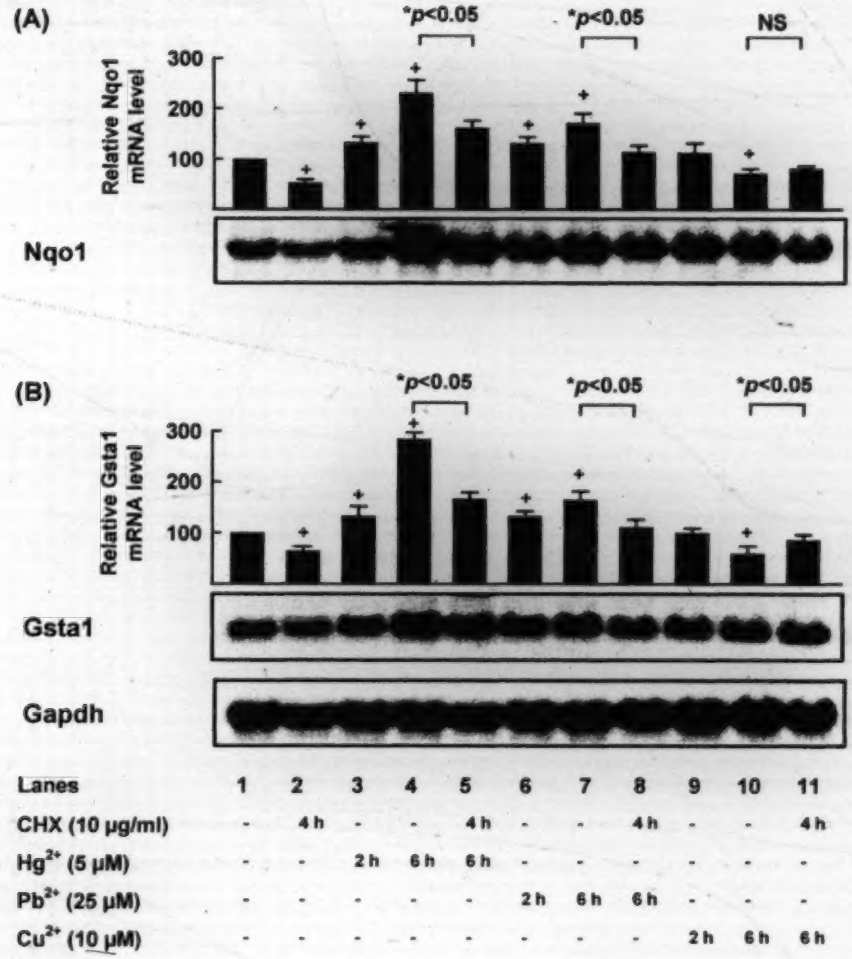
(A)
*p<0.05
*p<0.05
NS
Relative Nqo1 mRNA level
300
200
100
Nqo1
(B)
*p<0.05
*p<0.05
*p<0.05
Relative Gsta1 mRNA level
300
200
100
Gsta1
Gapdh
Lanes 1 2 3 4 5 6 7 8 9 10 11
CHX (10 μg/ml) - 4 h - - 4 h - - 4 h - - 4 h
Hg2+ (5 μM) - - 2 h 6 h 6 h - - - - - -
Pb2+ (25 μM) - - - - - 2 h 6 h 6 h - - -
Cu2+ (10 μM) - - - - - - - - 2 h 6 h 6 h

Figure 3.26. Mechanism of the inhibition of metal-mediated induction of (A) Nqo1 and (B) Gsta1 mRNA by a protein synthesis inhibitor. Hepa 1c1c7 cells were treated with 10 μg/ml CHX for 4 h, 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} for 2 or 6 h, or metals for 2 h followed by metals plus CHX for additional 4 h. Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gsta1 mRNA (mean ± SEM., n = 3), expressed as percent of the control. One of three representative experiments is shown. +, $p < 0.05$ compared with control; *, $p < 0.05$ compared with the same treatment for 6 h in the absence of CHX; and NS, not significant.

As shown in Fig. 3.26, CHX alone caused a significant decrease of the constitutive expression of Nqo1 and Gsta1 mRNAs after 4 h of exposure (lane 2). In addition, metals increased the expression of Nqo1 and Gsta1 mRNAs at 2 and 6 h in a time-dependent manner. On the other hand, the mRNA levels of either Nqo1 or Gsta1 from cells treated with metals for 6 h plus CHX for 4 h (lanes 5, 8, and 11) was almost similar to the mRNA levels obtained from cells treated with metals alone for 2 h (lanes 3, 6, and 9). These results imply that CHX did not affect the level of existing Nqo1 or Gsta1 mRNA; rather, it inhibited the *de novo* RNA synthesis, which is required by metals for the induction of these genes. This suggests that metals regulate the expression of Nqo1 and Gsta1 mRNAs at the transcriptional level.

3.3.6. Induction of Nqo1 and Gsta1 mRNAs by Metals is a Transcriptional Mechanism.

To further confirm the involvement of a transcriptional mechanism in the regulation of *Nqo1* and *Gsta1* genes by metals, we tested the hypothesis that inhibition of the proteasome-dependent degradation of Nrf2 by MG-132, a potent 26S proteasome inhibitor, would reverse the repressor effect of CHX on the metal-mediated induction of Nqo1 and Gsta1 mRNAs. Therefore, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence and absence of either 10 μg/ml CHX or 10 μg/ml CHX plus 25 μM MG-132 at a concentration of MG-132 known to inhibit the proteasome-dependent degradation of Nrf2 protein in Hepa 1c1c7 cells (Ma et al., 2004).

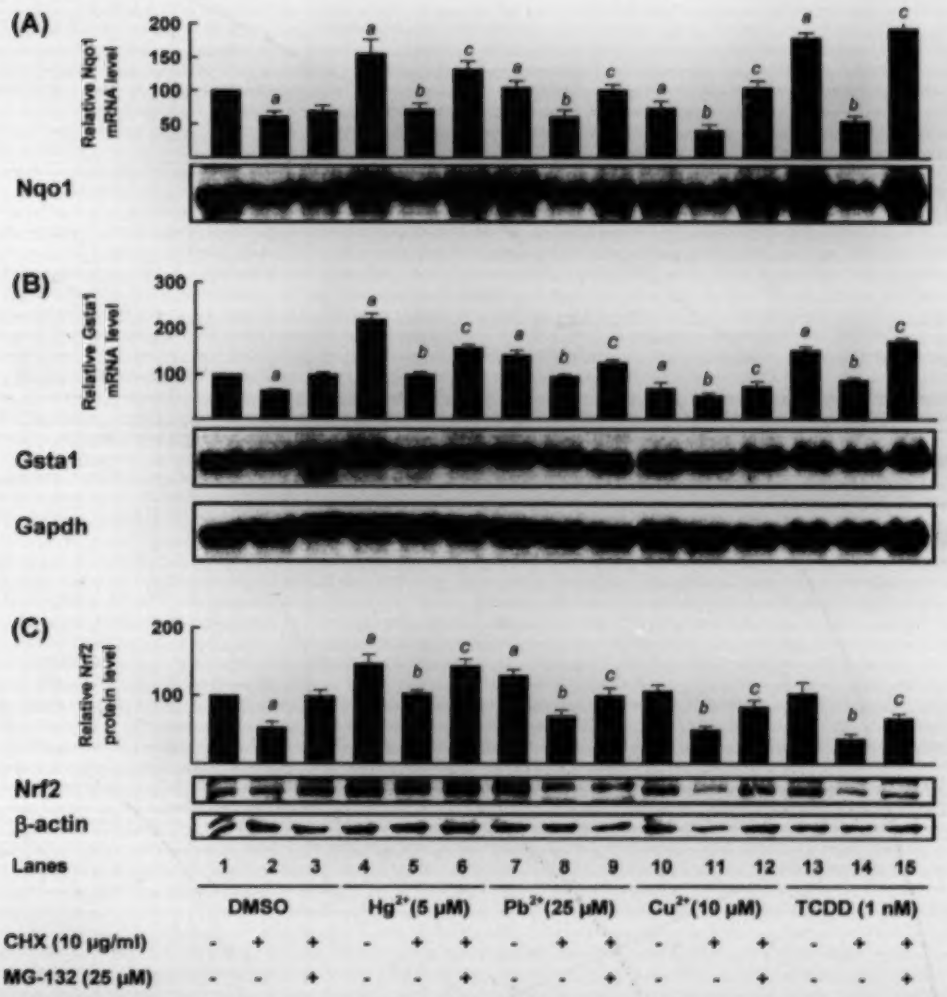

Figure 3.27. Effect of a 26S proteasome inhibitor on the inhibition of metal-mediated induction of (A) Nqo1, (B) Gsta1 mRNA, and (C) Nrf2 protein by CHX. Hepa 1c1c7 cells were treated for 6 h with 10 μg/ml CHX or MG-132, a 26S proteasome inhibitor, plus CHX for 30 min before exposure to 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD. A and B, Nqo1 and Gsta1 mRNA levels normalized to Gapdh mRNA levels were determined by Northern blot analysis. The graph represents the relative normalized amount of Nqo1 or Gst ya mRNA (mean ± SEM, $n = 3$), expressed as percent of the control. C, Nrf2 protein normalized to β-actin was determined by Western blot analysis. The graph represents the relative normalized amount of Nrf2 protein (mean ± S.E.M., $n = 3$), expressed as percent of the control. One of three representative experiments is shown. *a*, $p < 0.05$ compared with control; *b*, $p < 0.05$ compared with same treatment in the absence of CHX; and *c*, $p < 0.05$ compared with same treatment in the presence of CHX.

In Fig. 3.27A and B (lane 2), treatment of Hepa 1c1c7 cells with CHX alone caused a significant reduction in Nqo1 and Gsta1 mRNAs. MG-132 alone, in the absence of CHX, did not significantly alter the Nqo1 and Gsta1 mRNA expression (data not shown), which is consistent with previously published data in Hepa 1c1c7 cells (Ma et al., 2004). However, in the presence of CHX, MG-132 significantly reversed the CHX-mediated inhibition of Nqo1 and Gsta1 mRNAs to their control levels (lane 3). Importantly, treatment of the cells with metals plus CHX and MG-132 significantly reversed the CHX-mediated inhibition of the metal-inducible Nqo1 and Gsta1 mRNAs (lanes 6, 9, and 12). A similar result was observed with TCDD (lanes 13–15). Taken together, these results confirm that metals regulate the expression of *Nqo1* and *Gsta1* genes by a transcriptional mechanism.

3.3.7. Nrf2 Protein is Required for the Induction of Nqo1 and Gsta1 mRNAs by Metals.

To directly explore whether Nqo1 and Gsta1 mRNAs can be induced by metals in a Nrf2-dependent manner, Hepa 1c1c7 cells were treated for 6 h with 5 μM Hg^{2+}, 25 μM Pb^{2+}, 10 μM Cu^{2+}, or 1 nM TCDD in the presence and absence of either 10 μg/ml CHX or 10 μg/ml CHX plus 25 μM MG-132. Nrf2 protein expression was then detected by Western blot analysis.

Figure 3.27C shows that Hg^{2+} and Pb^{2+} significantly increased the level of Nrf2 compared with control, whereas Cu^{2+} and TCDD did not significantly alter Nrf2 level (Fig. 3.27C, lanes 1, 4, 7, 10, and 13). Furthermore, Hg^{2+}- and Pb^{2+}-mediated induction of Nqo1 and

Gsta1 mRNAs (Fig. 3.27A and B, lanes 4 and 7) was associated with elevated Nrf2 level (Fig. 3.27C, lanes 4 and 7), suggesting that a Nrf2-dependent mechanism is involved. As expected, treatment of the cells with CHX alone or CHX plus metals significantly decreased the constitutive (Fig. 3.27C, lane 2) and metal-inducible (Fig. 3.27C, lanes 5 and 8) Nrf2 expression that was associated with a decrease in Nqo1 and Gsta1 mRNA expressions (Fig. 3.27A and B, lanes 2, 5, 8, and 11). Importantly, the CHX-mediated decrease of Nrf2 protein, Nqo1 and Gsta1 mRNA levels were significantly reversed by MG-132 treatment (Fig. 3.27A–C, lanes 3, 6, 9, and 12). These results imply that Nrf2 is required by metals for the induction of Nqo1 and Gsta1 mRNAs.

3.3.8. Lack of a Post-Transcriptional Regulation of *Nqo1* and *Gsta1* Genes by Metals.

To further investigate whether the effects of metals on the Nqo1 and Gsta1 mRNAs could be attributed to a post-transcriptional stabilization of the mRNA, an Act-D chase experiment was performed to determine the effect of metals on the half-lives of both constitutive and inducible Nqo1 and Gsta1 mRNAs. If metals stabilize the mRNA, we should observe an increase in the mRNA half-life.

Act-D chase experiment revealed that the decay of Nqo1 and Gsta1 mRNA transcripts isolated from Hepa 1c1c7 cells pretreated with either vehicle or TCDD alone followed first-order kinetics, with approximate half-lives of 16.45 ± 2.52 and 10.90 ± 1.17 h (constitutive, Fig. 3.28A and B) and approximately 17.88 ± 0.48 and 11.4 ± 0.45 h (inducible, Fig. 3.29A and B). However, treatment of the cells with metals did not

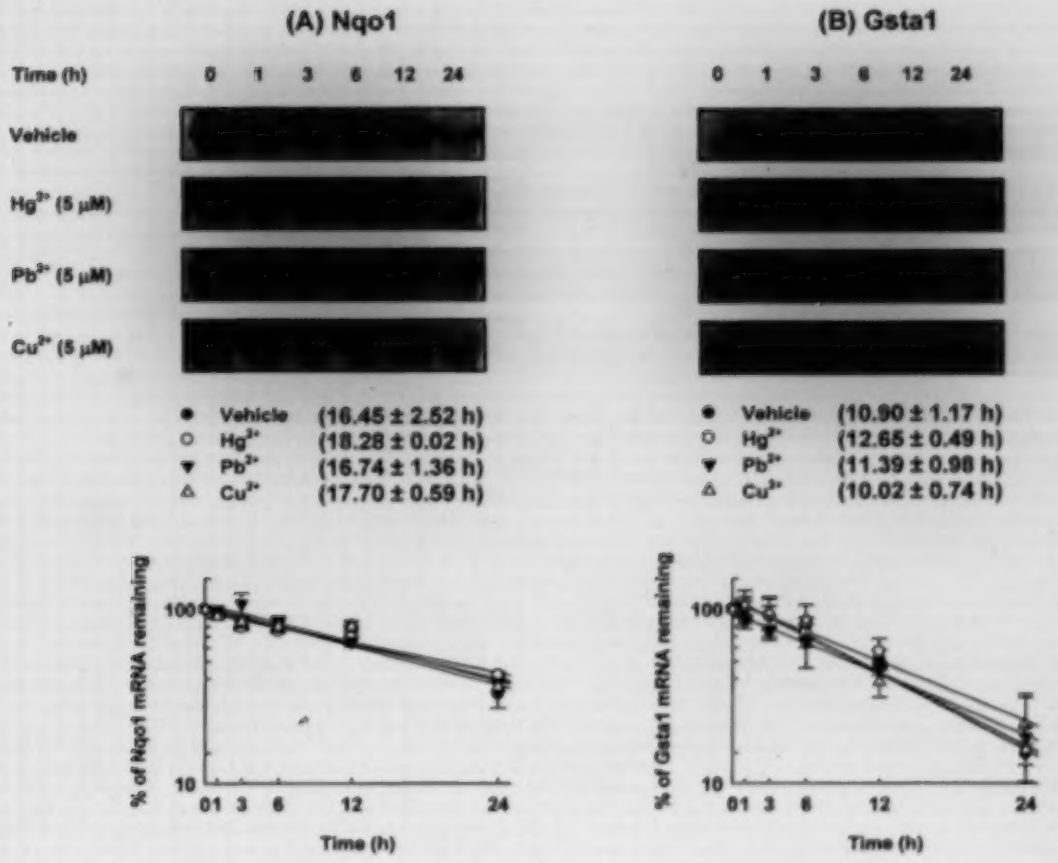

Figure 3.28. Effect of metals on the constitutive (A) Nqo1 and (B) Gsta1 mRNA half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, then incubated in fresh media containing vehicle, 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 5 μg/ml Act-D, a RNA synthesis inhibitor. Nqo1 and Gsta1 mRNAs normalized to Gapdh was determined by Northern blot analysis. All mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of mRNA amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). One of three representative experiments is shown.

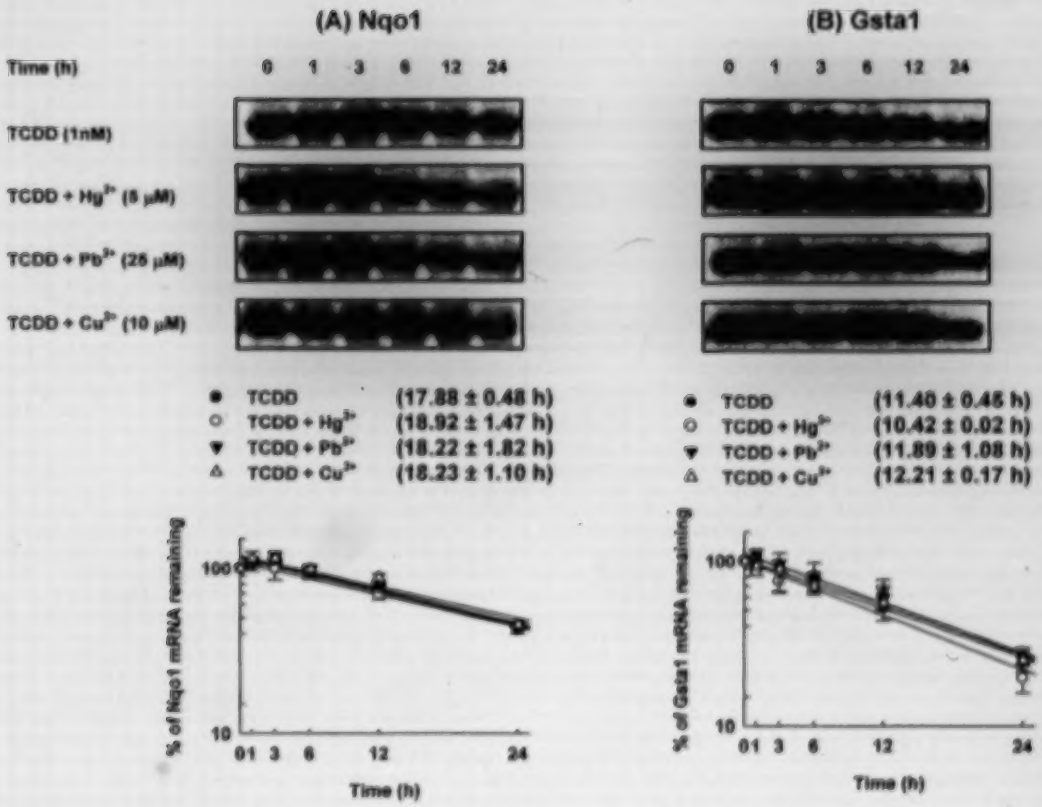
(A) Nqo1
(B) Gsta1
Time (h)
0 1 3 6 12 24
TCDD (1nM)
TCDD + Hg2+ (5 μM)
TCDD + Pb2+ (25 μM)
TCDD + Cu2+ (10 μM)
TCDD (17.88 ± 0.48 h)
TCDD + Hg2+ (18.92 ± 1.47 h)
TCDD + Pb2+ (18.22 ± 1.82 h)
TCDD + Cu2+ (18.23 ± 1.10 h)
TCDD (11.40 ± 0.45 h)
TCDD + Hg2+ (10.42 ± 0.02 h)
TCDD + Pb2+ (11.89 ± 1.08 h)
TCDD + Cu2+ (12.21 ± 0.17 h)
% of Nqo1 mRNA remaining
% of Gsta1 mRNA remaining
100
10
01 3 6 12 24
Time (h)

Figure 3.29. Effect of metals on the TCDD-inducible (A) Nqo1 and (B) Gsta1 mRNA half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then incubated with 1 nM TCDD for 12 h. The cells were then washed three times with PBS and incubated in fresh media containing 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 5 μg/ml Act-D, a RNA synthesis inhibitor. Nqo1 and Gsta1 mRNAs normalized to Gapdh was determined by Northern blot analysis. All mRNA decay curves were analyzed individually, and the half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of mRNA amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). One of three representative experiments is shown.

significantly affect the half-lives of Nqo1 and Gsta1 mRNAs of both vehicle- and TCDD-treated Hepa 1c1c7 cells, implying that all three metals did not change the stability of Nqo1 and Gsta1 mRNAs. These results revealed that the modulation of Nqo1 and Gsta1 mRNAs by metals is not regulated by a post-transcriptional mechanism.

3.3.9. Lack of a Post-Translational Regulation of *Nqo1* and *Gsta1* Genes by Metals.

The sustained elevation of Nqo1 and Gsta1 mRNAs with variable response to the catalytic activities (Fig. 3.23A and B) prompted further investigation to examine whether metals could modify the stability of Nqo1 and Gsta1 proteins. Therefore, the effect of metals on the constitutive and inducible Nqo1 and Gsta1 protein half-lives was determined using CHX chase experiments. Our results clearly showed that the half-lives of Nqo1 and Gsta1 proteins were determined to be greater than 24 h, at both the constitutive (Fig. 3.30A and B) and inducible (Fig. 3.31A and B) levels. Furthermore, the stability of both proteins did not appear to be significantly altered by metals up to 24 h (Figs. 3.30 and 3.31). The results of these experiments indicate a lack of a post-translational regulation of the Nqo1 and Gsta1 by metals.

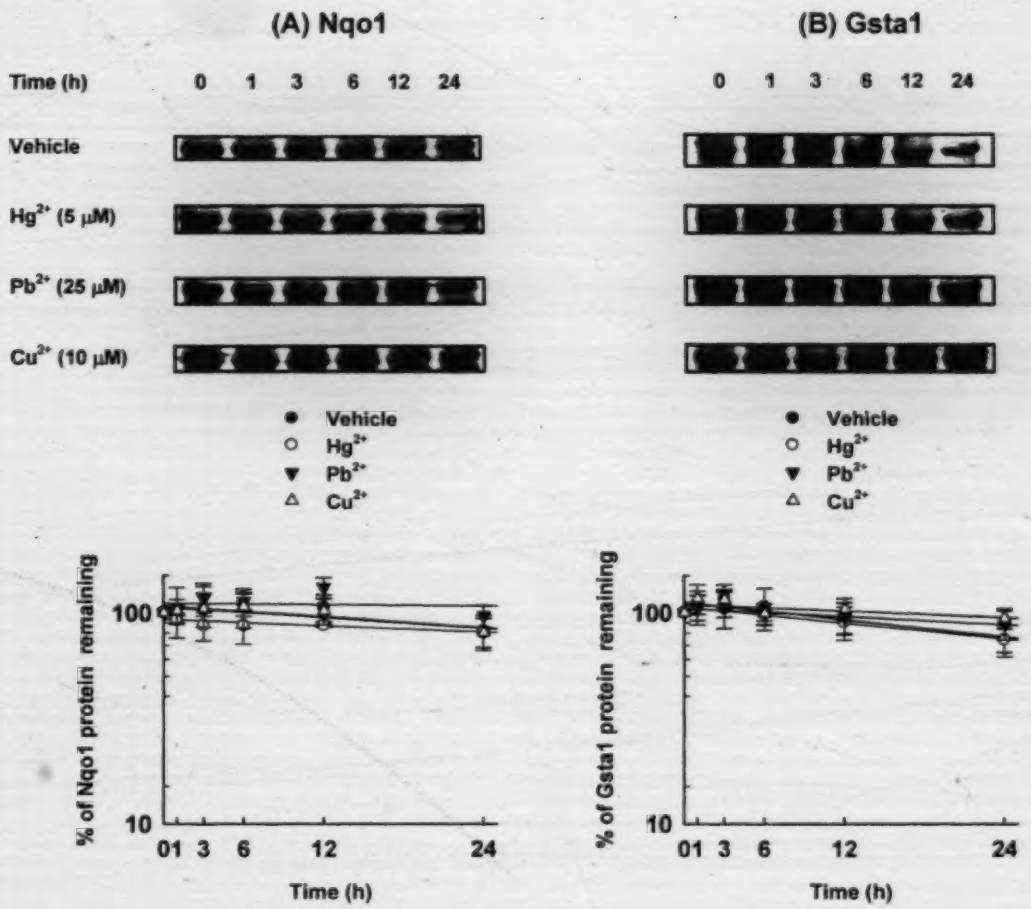

Figure 3.30. Effect metals on the constitutive (A) Nqo1 and (B) Gsta1 protein half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates, then incubated in fresh media containing 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 10 μg/ml CHX, a protein synthesis inhibitor. Nqo1 and Gsta1 proteins normalized to β-actin were determined by Western blot analysis. All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of protein amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time, The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). One of three representative experiments is shown.

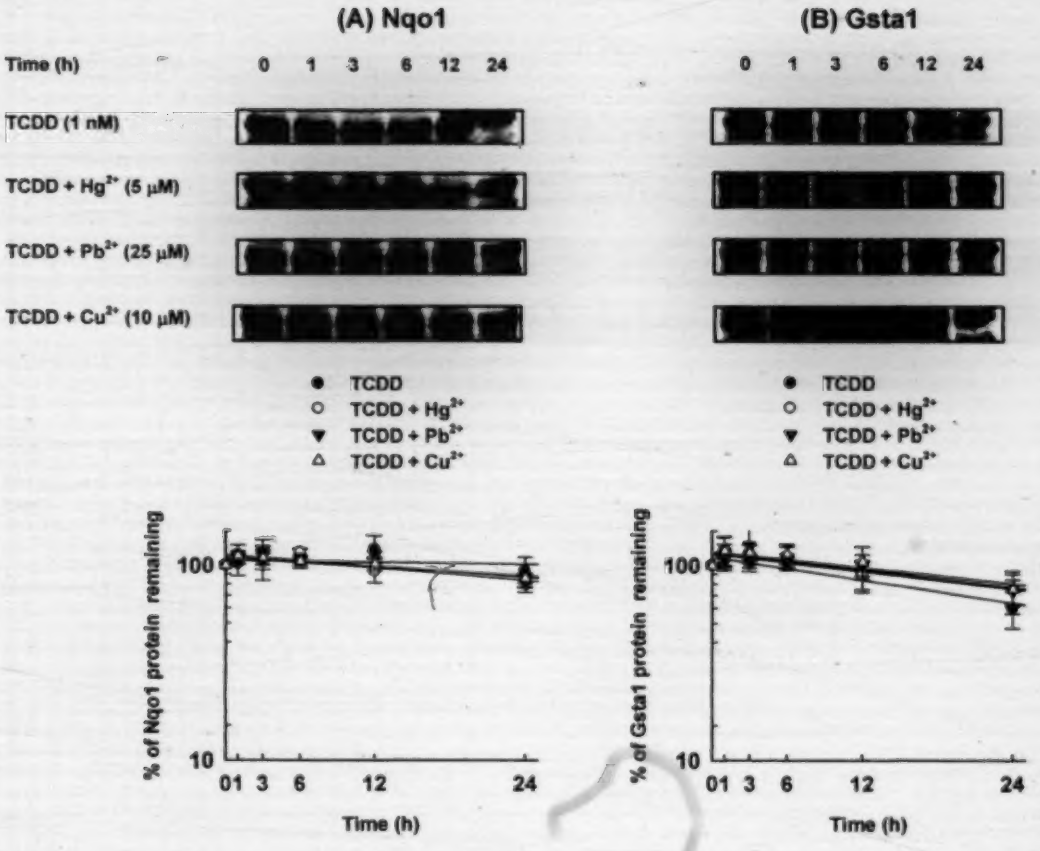

Figure 3.31. Effect of metals on the TCDD-inducible (A) Nqo1 and (B) Gsta1 protein half-lives. Hepa 1c1c7 cells were grown to 70% confluence in six-well cell culture plates and then incubated with 1 nM TCDD for 24 h. The cells were then washed three times with PBS and incubated in fresh media containing 5 μM Hg^{2+}, 25 μM Pb^{2+}, or 10 μM Cu^{2+} plus 10 μg/ml CHX, a protein synthesis inhibitor. Nqo1 and Gsta1 proteins normalized to β-actin were determined by Western blot analysis. All protein decay curves were analyzed individually. The half-life was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of protein amount, expressed as a percent of treatment at $t = 0$ (maximum, 100%) level, versus time, The half-lives obtained from three independent experiments were then used to calculate the mean half-life (mean ± SEM, $n = 3$). One of three representative experiments is shown.

3.4. THE ROLE OF REDOX-SENSITIVE TRANSCRIPTION FACTORS, NF-κB AND AP-1 IN THE MODULATION OF THE *Cyp1a1* GENE BY Hg^{2+}, Pb^{2+}, AND Cu^{2+}

3.4.1. Hg^{2+}, Pb^{2+}, and Cu^{2+} Induce Oxidative Stress in Hepa 1c1c7 Cells

To determine the capacity of metals to alter redox status of Hepa 1c1c7 cells, cellular oxidative stress markers, HO-1 mRNA levels and ROS production, were measured following 24 h treatment of Hepa 1c1c7 with increasing concentrations of Hg^{2+} (2.5-10 μM), Pb^{2+} (10-100 μM), and Cu^{2+} (1-100 μM). Figure 1A shows that all the metals tested were able to increase HO-1 mRNA levels in a concentration-dependent manner. At the highest concentrations tested, Cu^{2+} was the most potent inducer (30-fold) followed by Hg^{2+} and Pb^{2+} (approximately 15- and 12-fold, respectively) (Fig. 3.32A).

In addition, all the metals significantly increased ROS production in a metal- and substrate-dependent manner. The extent of DCF-DA oxidation by ROS to its fluorescent dye was significantly increased with Hg^{2+} and Cu^{2+}, but not Pb^{2+}, by approximately 35% and 250%, respectively (Fig. 3.32B). Furthermore, oxidation of DHR-123, another marker for ROS production, particularly hydroxyl radical (OH) (Crow, 1997; Shankar et al., 2003), to rhodamine was increased with Pb^{2+} and Cu^{2+}, but not Hg^{2+}, by approximately 40% and 60%, respectively (Fig. 3.32B).

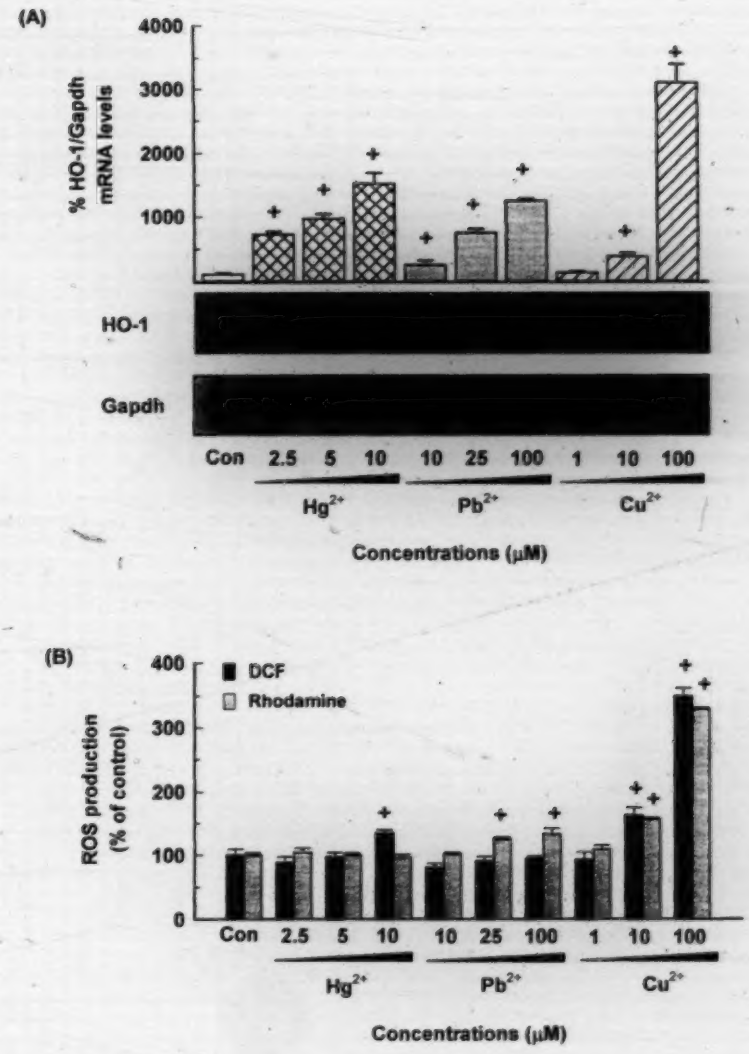

Figure 3.32. Effects of metals on HO-1 mRNA level (A) and ROS production (B). (A) Hepa 1c1c7 cells were treated for 6 h with various concentrations of Hg^{2+} (2.5-10 μM), Pb^{2+} (10-100 μM), and Cu^{2+} (1-100 μM). HO-1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. $^{+}p< 0.05$ compared to control (Con). (B) Hepa 1c1c7 cells treated for 24 h with various concentrations of metals were incubated with DCF-DA (10 μM) or DHR-123 (100 μM) for 30 min. DCF and rhodamine formation was measured flurometrically using excitation/emission wavelengths of 484/535. Values are presented as mean ± SEM, n=8. $^{+}p<0.05$ compared to corresponding control (Con).

3.4.2. Depletion of GSH Modulates the Expression of *HO-1* and *Cyp1a1* Genes by Metals

To further explore the effect of the antioxidant GSH on the modulation of *HO-1* and *Cyp1a1* genes by metals, we chemically depleted cellular GSH contents using BSO, a γ-glutamylcysteine synthetase inhibitor (Shertzer et al., 1995). Our results showed that exposure of Hepa 1c1c7 cells to BSO (250 μM) alone for at least 18-24 h induced basal HO-1 mRNA level (3-fold) and further potentiated only the Pb^{2+}-mediated HO-1 mRNA induction, whereas no significant changes were observed with the other metals or TCDD treatment (Fig. 3.33A). This was associated with a significant potentiation of Cyp1a1 mRNA induction by Pb^{2+} (Fig 3.33B).

In an attempt to explore whether or not GSH is directly involved in the inhibitory effects of metals on the TCDD-induced Cyp1a1 activity, Hepa 1c1c7 cells were pretreated for 12 h with BSO prior to the addition of a mixture of TCDD plus metals. As shown in Fig. 3.33C, BSO treatment did not significantly alter the Cyp1a1 enzyme activity in either untreated- and TCDD-treated cells. However, BSO further potentiated the inhibitory effects of the three metals on the TCDD-mediated Cyp1a1 induction; Pb^{2+} was the most potent followed by Cu^{2+} and Hg^{2+}. These results demonstrated that increased cellular oxidative stress significantly modulates the expression of *Cyp1a1* gene by metals.

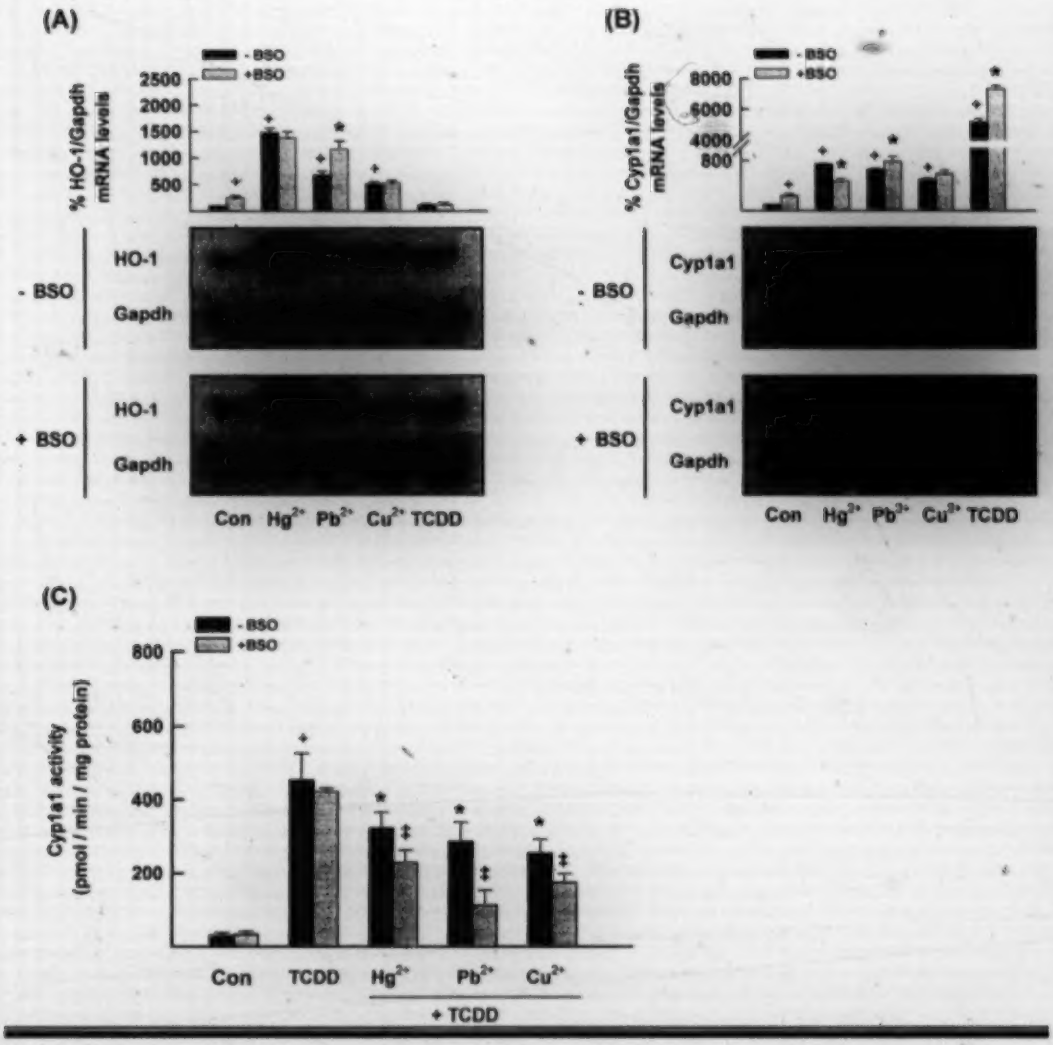

Figure 3.33. Effect of GSH depletion on metal-mediated induction of HO-1 mRNA (A), Cyp1a1 mRNA (B) and Cyp1a1 activity (C). (A and B) Hepa 1c1c7 cells were pretreated for 24 h with BSO (250 μM), a GSH synthesis inhibitor, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n = 3), expressed as percent of the control. Only one of three representative experiments is shown. $^{+}p<0.05$, compared to control (Con); and $^{*}p<0.05$, compared to corresponding - BSO treatments. (C) Hepa 1c1c7 cells were pretreated for 12 h with BSO (250 μM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{+}p<0.05$ compared to control (Con); $^{*}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding -BSO treatments.

3.4.3. Hg^{2+}, Pb^{2+}, and Cu^{2+} Increased NF-κB and AP-1 Binding Activity in Hepa 1c1c7 Cells

To investigate whether metal-induced oxidative stress triggers NF-κB and AP-1 activation, we tested the capacity of metals to increase nuclear NF-κB and AP-1 binding activity. Figure 3.34 shows that the positive control, LPS, significantly increased NF-κB and AP-1 DNA binding activity (lanes 5 and 11) as compared to control (lanes 1 and 7), respectively. On the other hand, all metals were able to induce the binding activity of NF-κB (lanes 2, 3, and 4) and AP-1 (lanes 8, 9, and 10) to their responsive elements in a metal-dependent manner. Densitometry scanning showed that Cu^{2+} was the most potent inducer of NF-κB DNA binding activity (5-fold, lane 4), whereas Pb^{2+} caused the highest induction of AP-1 activity (4-fold, lane 9). Furthermore, incubation with a 100-fold molar excess of competitor oligonucleotides resulted in diminished binding activity (lane 6 and 12, respectively), indicating the specificity of binding.

3.4.4. Activation of NF-κB Signaling Pathway Suppresses Metals-Mediated Effects on *Cyp1a1* Gene Expression

To determine whether NF-κB is directly involved in the modulation of HO-1 mRNA and Cyp1a1 mRNA and activity levels by metals, we tested the effect of the NF-κB specific chemical activator, PMA.

Treatment of cells with PMA (25 nM) significantly suppressed the induction of HO-1 mRNA by the three metals (Fig. 3.35A). This was associated with a complete inhibition of metal-mediated induction of Cyp1a1 mRNA, in a manner similar to what was

observed with TCDD (Fig. 3.35B). At the activity level, pretreatment of cells with PMA significantly reduced the basal and TCDD-induced Cyp1a1 activity levels by approximately 25% and 40%, respectively (Fig. 3.35C). However, the inhibitory effects of metals on the TCDD-mediated Cyp1a1 induction were further potentiated by PMA treatment (Fig. 3.35C). This was most noticeable with Cu^{2+} (33%), followed by Hg^{2+} (27%) and Pb^{2+} (23%) treatments.

3.4.5. Inhibition of NF-κB Signaling Pathway Potentiates Metal-Mediated Effects on *Cyp1a1* Gene Expression

To further confirm the negative regulatory role of NF-κB, we tested the effect of the NF-κB inhibitor, PDTC. Figure 3.36A shows that although pretreatment of the cells with PDTC (10 μM) did not significantly alter HO-1 mRNA levels of either untreated or TCDD-treated cells, it further potentiated the increase of HO-1 mRNA levels by the three metals by approximately 6-8 fold. In contrast to the PMA data, PDTC further potentiated Hg^{2+}- and Cu^{2+}-mediated Cyp1a1 induction at the mRNA level (Fig. 3.36B). A similar pattern of expression was observed with TCDD. On the contrary, PDTC completely inhibited the Pb^{2+}-mediated Cyp1a1 mRNA induction (Fig. 3.36B).

At the activity level, cotreatment of cells with PDTC further potentiated Cyp1a1 activity levels by TCDD treatment, whereas significantly reversed the inhibitory effects of both Hg^{2+} (30%) and Pb^{2+} (23%), but not Cu^{2+}, on the TCDD-dependent induction of Cyp1a1 activity (Fig. 3.36C).

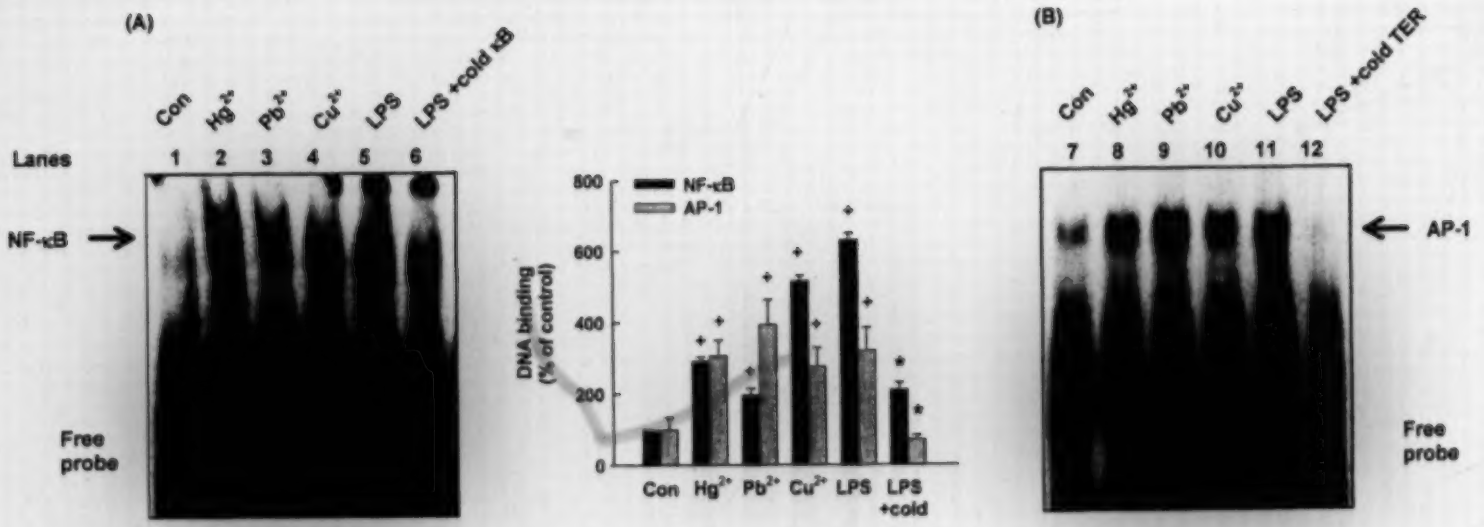

Figure 3.34. Effect of metals on NF-κB (A) and AP-1 (B) DNA binding. Nuclear extracts from Hepa 1c1c7 cells treated for 2 h with Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or LPS (5 μg/ml) were mixed with ^{32}P-labelled κB and TRE, and the formation of NF-κB (A) and AP-1 (B) DNA binding complexes was analyzed by EMSA. The specificity of binding was determined by incubating the protein treated with LPS with a 100-fold molar excess of cold probes. The arrow indicates the specific shift representing the DNA binding complex. This pattern of NF-κB and AP-1 activation was observed in three separate experiments, and only one is shown. The graph represents the relative DNA binding expressed as % of the control. $^{+}p<0.05$ compared to corresponding control (Con) and $^{*}p<0.05$, compared to corresponding LPS treatment.

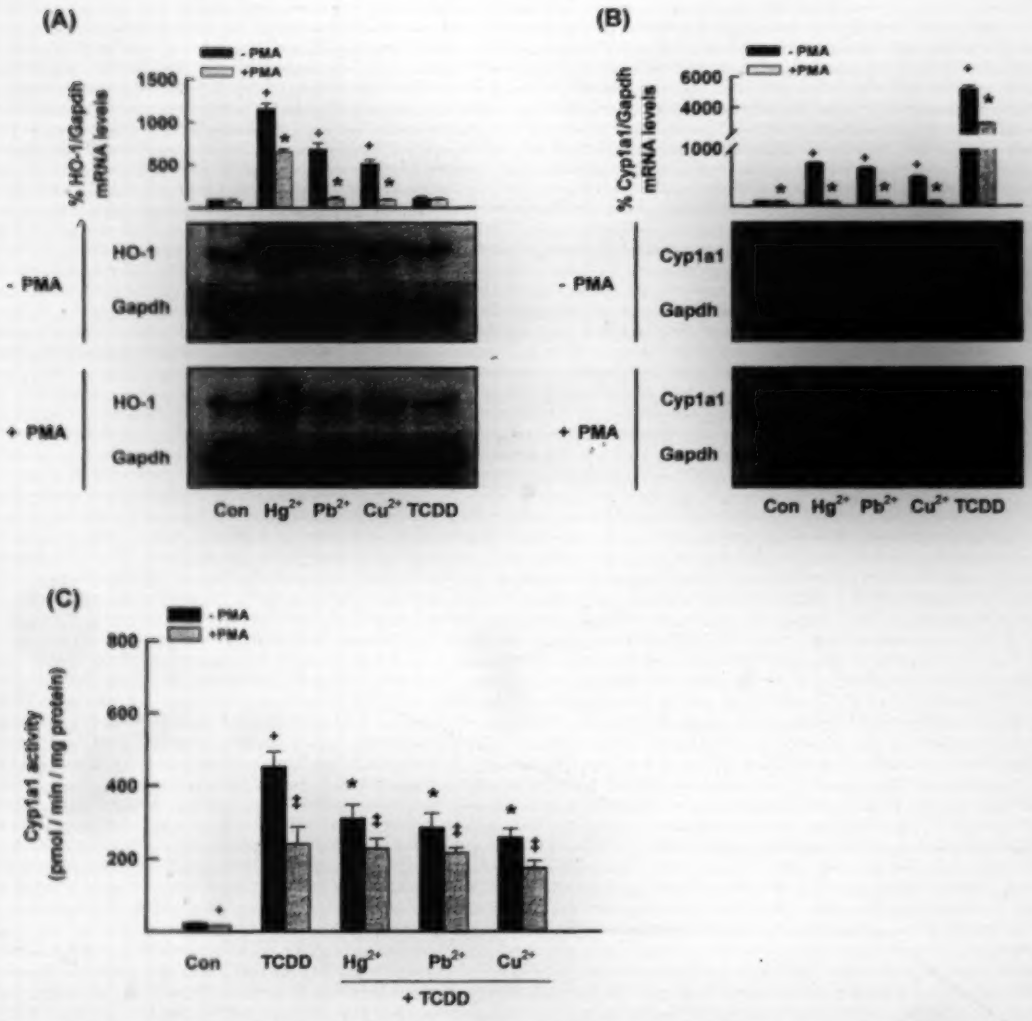

Figure 3.35. Effect of activation of NF-κB signaling pathway on the modulation of *HO-1* (A) and *Cyp1a1* genes (B and C) by metals. (A and B) Hepa 1c1c7 cells were pretreated for 24 h with PMA (25 nM), a NF-κB activator, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n = 3), expressed as percent of the control. Only one of three representative experiments is shown. $^{+}p<0.05$, compared to control (Con); and $^{*}p<0.05$, compared to corresponding - PMA treatments. (C) Hepa 1c1c7 cells were pretreated for 2 h with PMA (25 nM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{+}p<0.05$ compared to control (Con); $^{*}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding -PMA treatments.

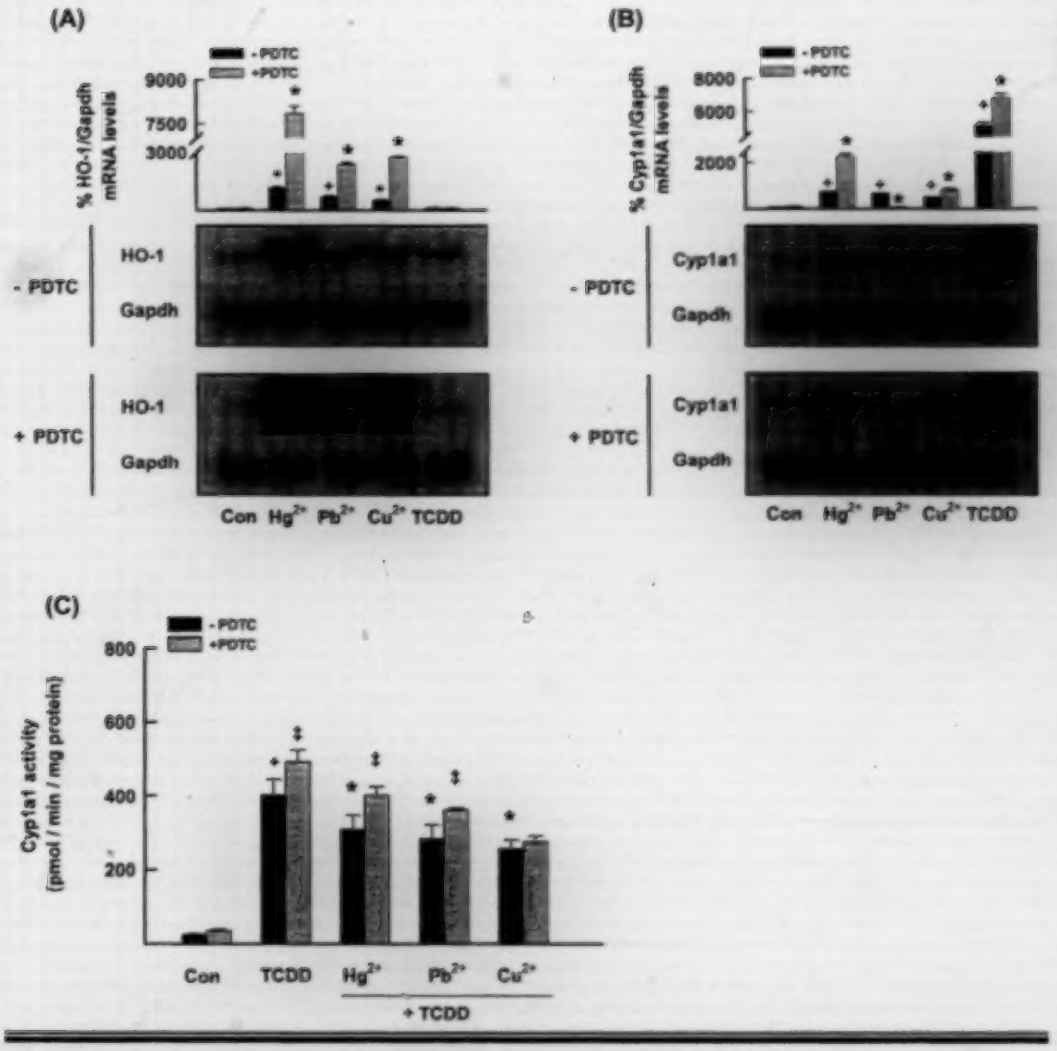

Figure 3.36. Effect of inhibition of NF-κB signaling pathway on the modulation of *HO-1* (A) and *Cyp1a1* genes (B and C) by metals. (A and B) Hepa 1c1c7 cells were pretreated for 24 h with PDTC (10 μM), a NF-κB inhibitor, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n=3), expressed as percent of the control. Only one of three representative experiments is shown. $^{+}p<0.05$, compared to control (Con); and $^{*}p<0.05$, compared to corresponding - PDTC treatments. (C) Hepa 1c1c7 cells were pretreated for 2 h with PDTC (10 μM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{+}p<0.05$ compared to control (Con); $^{*}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding -PDTC treatments.

3.4.6. The role of AP-1 Signaling Pathway on the Modulation of *Cyp1a1* Gene Expression by Metals

To investigate the role of AP-1 activation on the modulation of *Cyp1a1* gene by metals, cells were pretreated for 2 h with the JNK inhibitor, SP600125 (10 μM), the ERK inhibitor, U0126 (10 μM), and the p38 MAPK inhibitor, SP203580 (10 μM) prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), and TCDD (1 nM) for indicated time periods. If these MAPK activities are critical, their inhibition would prevent metal-mediated effects on *Cyp1a1* gene expression.

3.4.6.1. Inhibition of JNK Signaling Pathway Suppresses the Metals-Mediated Effects on Cyp1a1 Gene Expression

Treatment of Hepa 1c1c7 cells with SP600125 (10 μM), a concentration known to block JNK signaling cascade and subsequently AP-1 activity (Chen et al., 2006), significantly suppressed both basal and metal-mediated induction of HO-1 mRNA levels (Fig. 3.37A). This was accompanied by a complete inhibition of all metal-mediated induction of Cyp1a1 mRNA. Similarly, SP600125 significantly inhibited the TCDD-mediated Cyp1a1 mRNA induction (Fig. 3.37B). On the other hand, SP600125 significantly suppressed the induction of Cyp1a1 activity by TCDD and further potentiated the inhibitory effects of metals on TCDD-mediated induction of Cyp1a1 activity (Fig. 3.37C). This effect was most notable with Pb^{2+} followed by Cu^{2+} and Hg^{2+}.

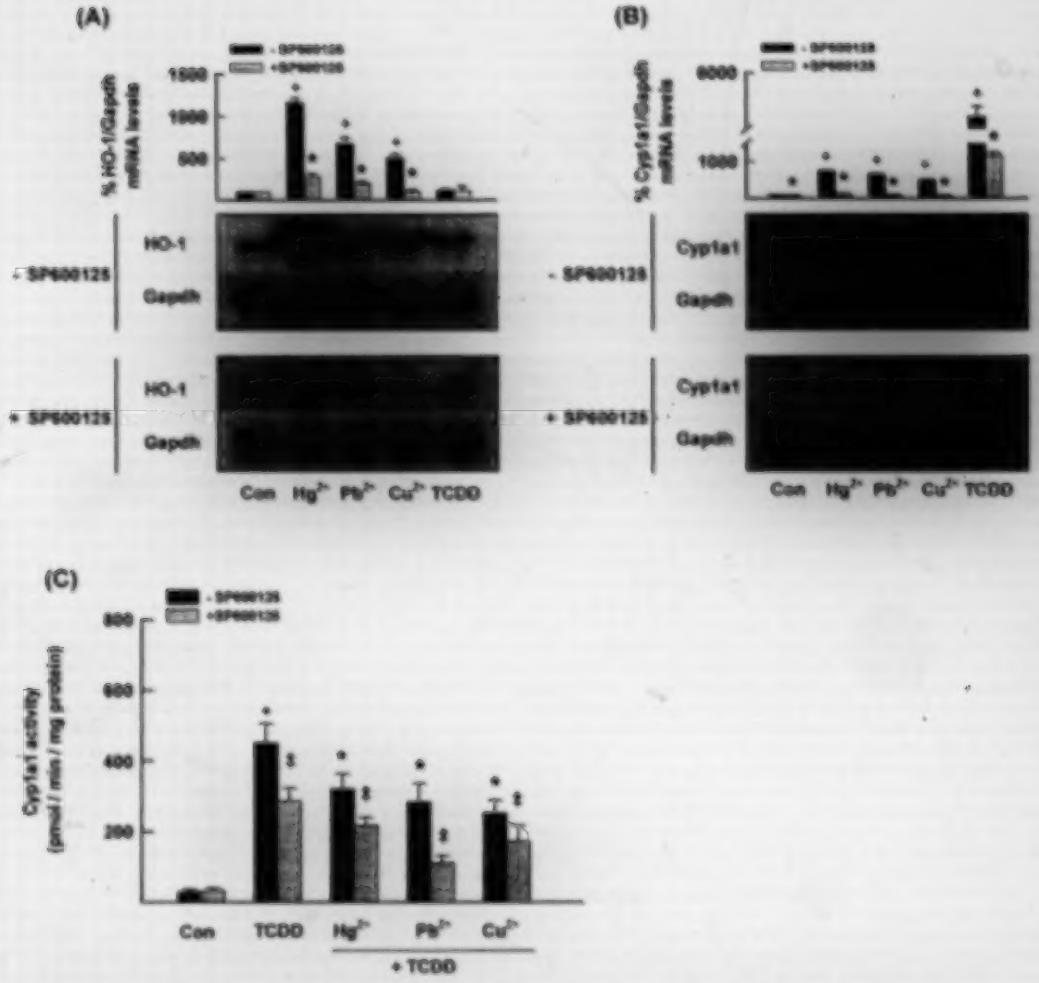

Figure 3.37. Effect of inhibition JNK signaling pathway on the modulation of *HO-1* (A) and *Cyp1a1* genes (B and C) by metals. (A and B) Hepa 1c1c7 cells were pretreated for 24 h with SP600125 (10 μM), a JNK inhibitor, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n = 3), expressed as percent of the control. Only one of three representative experiments is shown. $^{*}p<0.05$, compared to control (Con); and $^{\ddagger}p<0.05$, compared to corresponding - SP600125 treatments. (C) Hepa 1c1c7 cells were pretreated for 2 h with SP600125 (10 μM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{*}p<0.05$ compared to control (Con); $^{\ddagger}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding - SP600125 treatments.

3.4.6.2. *Inhibition of ERK Signaling Pathway Modulates the Metal-Mediated Effects on Cyp1a1 Gene Expression*

The ERK signaling pathway inhibitor, U0126, did not significantly alter basal HO-1 mRNA levels, but further potentiated only the Hg^{2+}-mediated induction of HO-1 mRNA by approximately 40%, and inhibited Pb^{2+}- and Cu^{2+}-mediated induction (Fig. 3.38A). On the other hand, U0126 pretreatment increased constitutive and metal-mediated Cyp1a1 mRNA induction, but suppressed TCDD-mediated induction (Fig. 3.38B). In a manner similar to the mRNA data, U0126 increased both constitutive and TCDD-inducible Cyp1a1 activity levels (Fig. 3.38C). Moreover, U0126 completely reversed the metal-mediated inhibition of TCDD-induced Cyp1a1 activity (Fig. 3.38C).

3.4.6.3. *Inhibition of p38 MAPK Signaling Pathway Modulates the Metals-Mediated Effects on Cyp1a1 Gene Expression*

Inhibition of p38 MAPK signaling pathway by SB203580 significantly inhibited the metal-mediated induction of HO-1 mRNA (Fig. 3.39A). This was associated with a complete suppression of all metal-mediated induction of Cyp1a1 mRNA in a manner similar to TCDD treatment (Fig. 3.39B). On the other hand, although SB203580 alone significantly induced Cyp1a1 activity by approximately 4-fold, it suppressed the TCDD-mediated induction (30%), but did not significantly alter the inhibitory effects of the metals on the TCDD-mediated induction of Cyp1a1 at the activity level (Fig. 3.39C).

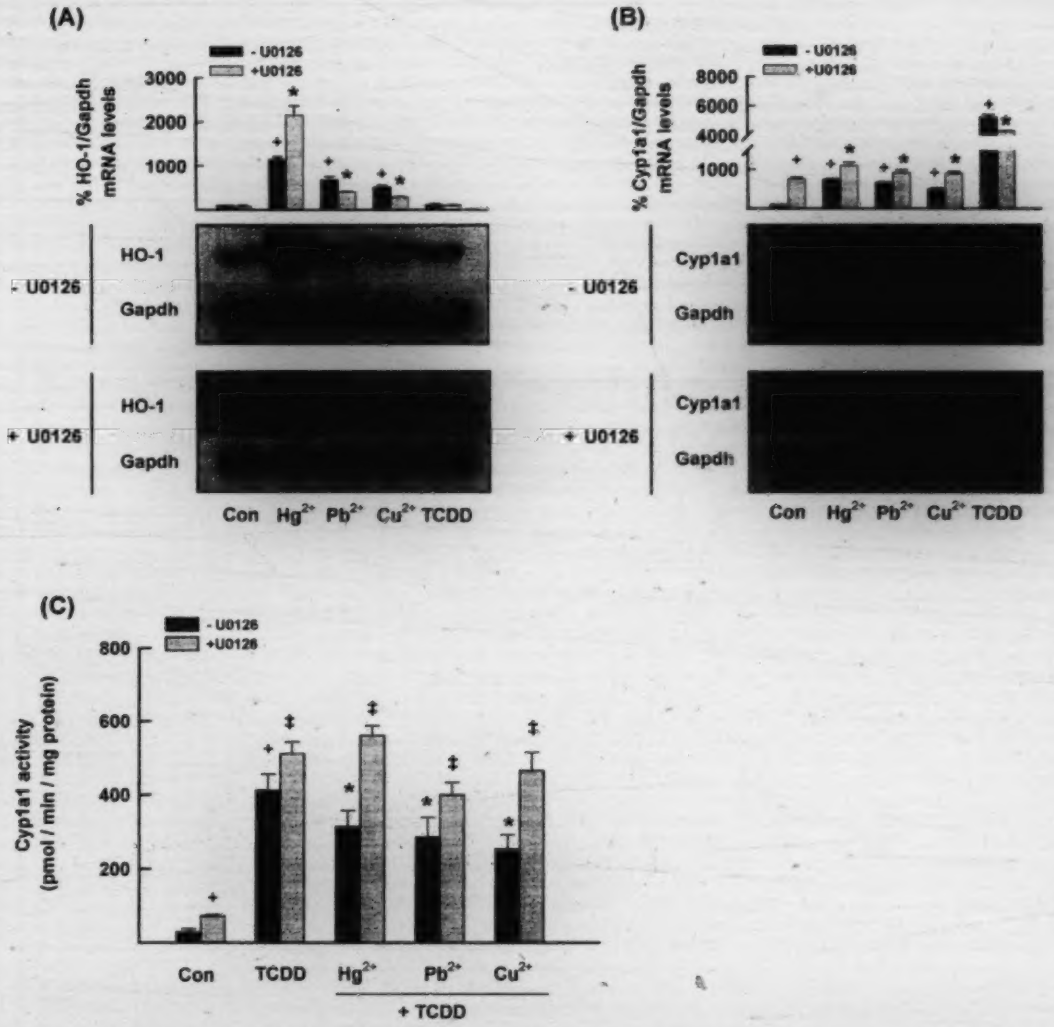

Figure 3.38. Effect of inhibition of ERK signaling pathway on the modulation of *HO-1* (A) and *Cyp1a1* genes (B and C) by metals. (A and B) Hepa 1c1c7 cells were pretreated for 24 h with U0126 (10 μM), an ERK inhibitor, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n = 3), expressed as percent of the control. Only one of three representative experiments is shown. $^{+}p<0.05$, compared to control (Con); and $^{*}p<0.05$, compared to corresponding - U0126 treatments. (C) Hepa 1c1c7 cells were pretreated for 2 h with U0126 (10 μM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{+}p<0.05$ compared to control (Con); $^{*}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding - U0126 treatments.

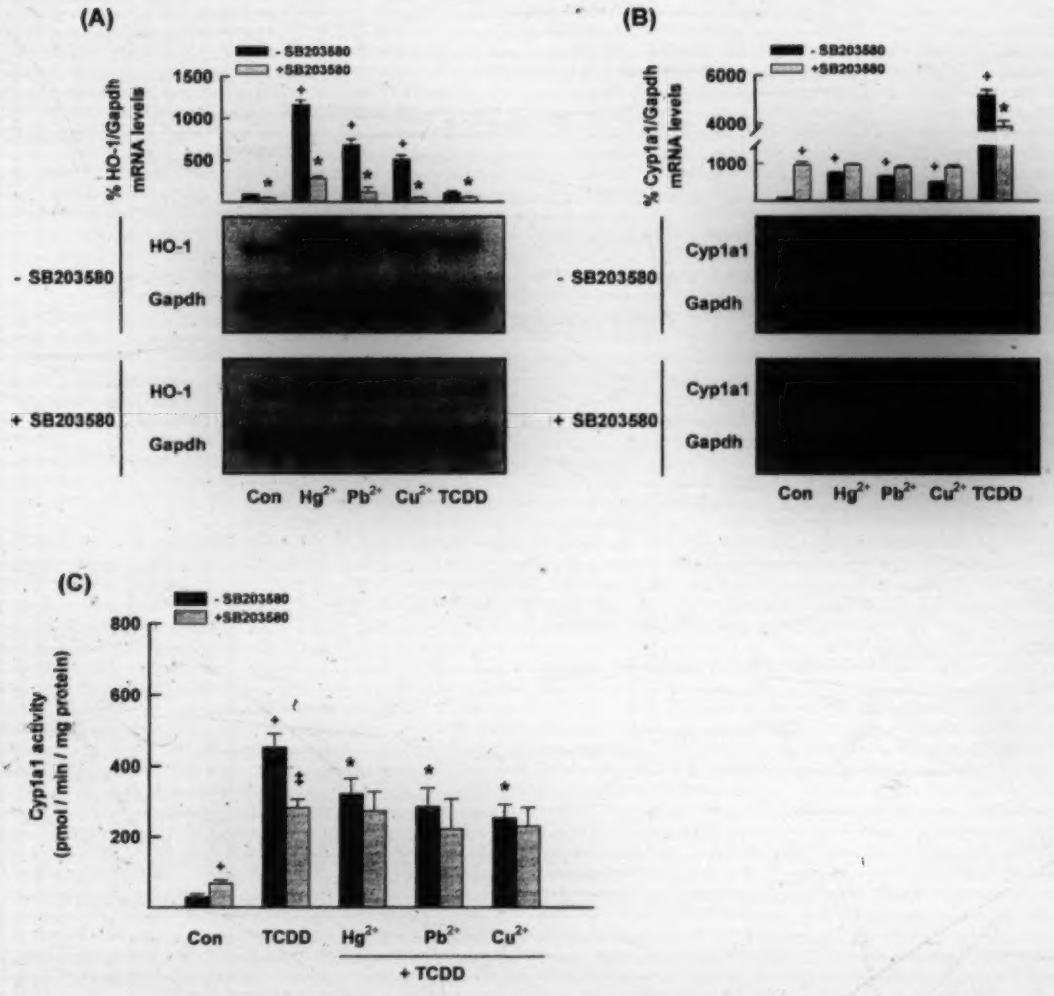

Figure 3.39. Effect of inhibition of p38 MAPK signaling pathway on the modulation of *HO-1* (A) and *Cyp1a1* genes (B and C) by metals. (A and B) Hepa 1c1c7 cells were pretreated for 24 h with SB203580 (10 μM), a p38 MAPK inhibitor, prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. HO-1 and Cyp1a1 mRNAs normalized to Gapdh were determined by Northern blot analysis. The graph represents the relative normalized amount HO-1 and Cyp1a1 mRNAs (mean ± S.E.M., n = 3), expressed as percent of the control. Only one of three representative experiments is shown. $^{+}p<0.05$, compared to control (Con); and $^{*}p<0.05$, compared to corresponding -SB203580 treatments. (C) Hepa 1c1c7 cells were pretreated for 2 h with SB203580 (10 μM) prior to the addition of TCDD (1 nM) in the presence and absence of metals for an additional 24 h. Cyp1a1 enzyme activity (mean ± SEM, n=8) was measured using 7ER as a substrate. $^{+}p<0.05$ compared to control (Con); $^{*}p<0.05$, compared to TCDD treatment. $^{\ddagger}p<0.05$, compared to corresponding - SB203580 treatments.

3.5. THE ROLE OF NF-κB AND AP-1 IN THE MODULATION OF THE *Nqo1* GENE BY Hg^{2+}, Pb^{2+}, AND Cu^{2+}

3.5.1. GSH Depletion Potentiates the Induction of the *Nqo1* Gene by Metals

To examine whether or not depletion of cellular GSH using BSO, a γ-glutamylcysteine synthetase inhibitor, would potentiate the induction of *Nqo1* by metals, Hepa 1c1c7 cells were pre-treated with BSO (250 μM), a concentration that shown to markedly deplete GSH levels in Hepa 1c1c7 cells (Shertzer et al., 1995), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), and Cu^{2+} (10 μM) for the indicated time points.

Exposure of the cells to BSO (250 μM) significantly increased the constitutive expression of Nqo1 at mRNA and activity levels by approximately 50% and 30%, respectively (Fig. 3.40A and b). Treatment of the cells with Hg^{2+} and Pb^{2+} alone significantly induced Nqo1 at mRNA and activity levels in a manner similar to what was observed with TCDD treatment (Fig. 3.40). Cu^{2+}, on the other hand, decreased Nqo1 mRNA whereas increased its catalytic activity. However, co-treatment with BSO and metals further potentiated the induction of Nqo1 mRNA levels by all three metals (Fig. 3.40A). At the activity levels, BSO further potentiated the induction mediated by Hg^{2+} and Cu^{2+} only (Fig. 3.40B). These results strongly suggest that Nqo1 induction by metals is mediated through an oxidative stress-dependent mechanism.

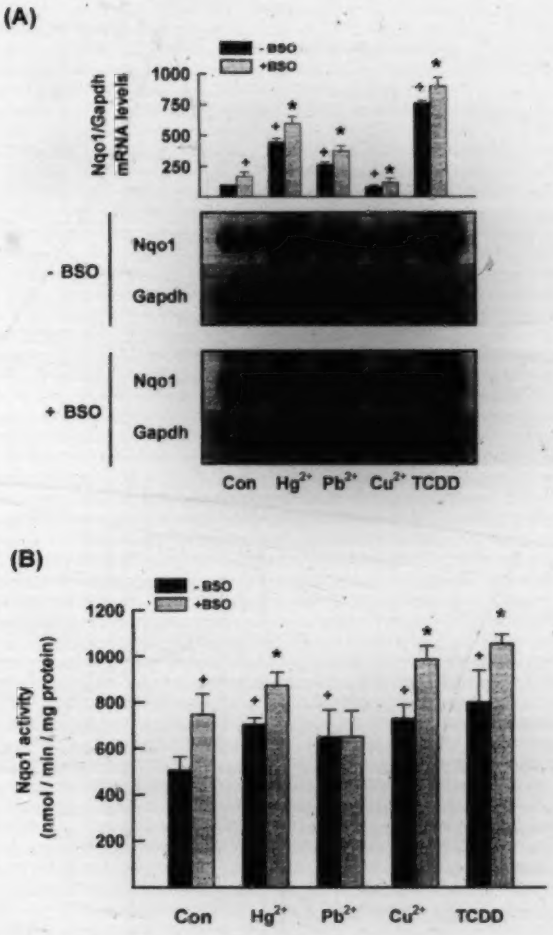

Figure 3.40. Effect of the prooxidant BSO on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 24 h with BSO (250 μM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding -BSO treatments.

3.5.2. NF-κB Negatively Regulates the Induction of *Nqo1* by Metals

To further determine the role of the redox-sensitive transcription factor, NF-κB, in the induction of *Nqo1* by metals, we tested the effect of an NF-κB specific chemical activator, PMA, and inhibitor, PDTC. For this purpose, Hepa 1c1c7 cells were pre-treated for 2 h with either PMA (25 nM) or PDTC (10 μM) prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), and TCDD (1 nM).

Treatment of cells with PMA significantly suppressed both the constitutive and metal-mediated induction of Nqo1 at mRNA and activity levels in a manner similar to what was observed with TCDD (Fig. 3.41A and B). The inhibitory effect of PMA was more pronounced at the mRNA than activity levels. Hg^{2+}- and Pb^{2+}-mediated Nqo1 mRNA induction was markedly inhibited by approximately 75% with PMA co-treatment, whereas only 25-30% inhibition was observed at the activity level.

In contrast to the PMA effect, treatment of Hepa 1c1c7 cells with the NF-κB inhibitor, PDTC, significantly increased the constitutive Nqo1 mRNA and enzyme activity levels (Fig. 3.42). However, differential effects were observed when cells were co-treated with PDTC and metals. PDTC treatment inhibited Hg^{2+}- whereas further potentiated Pb^{2+}-mediated induction of Nqo1 mRNA (Fig. 3.42A). On the other hand, PDTC did not alter Cu^{2+}- and TCDD-mediated effects. At the activity level, PDTC further potentiated the Hg^{2+}-mediated induction only, but did not significantly alter the induction of Nqo1 enzyme activity by other metals and TCDD (Fig. 3.42B).

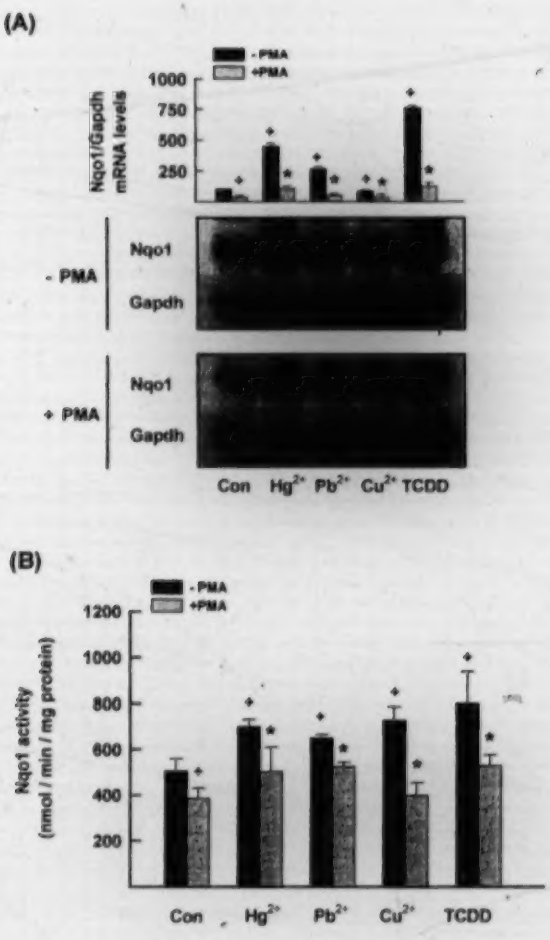

Figure 3.41. Effect of activation of the NF-κB signalling pathway on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 2 h with PMA, the NF-κB activator (25 nM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding -PMA treatment.

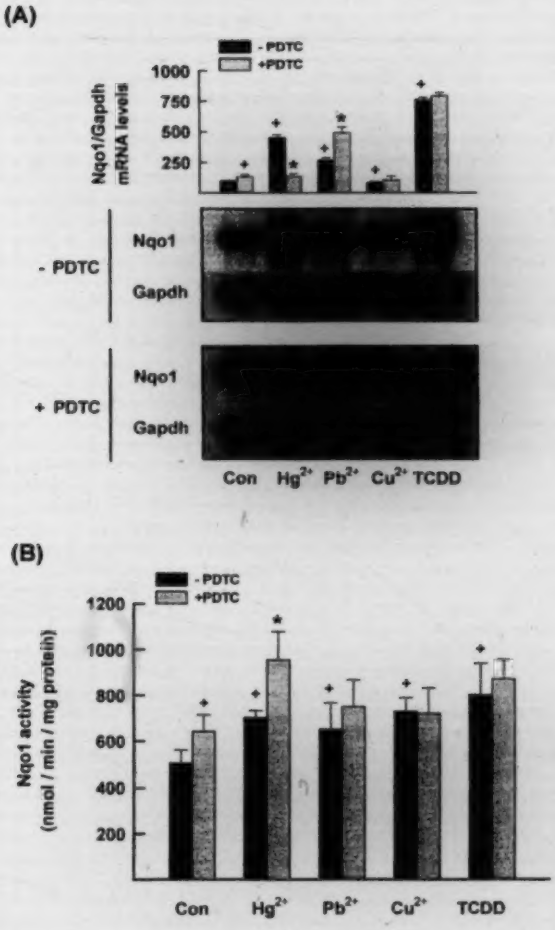

Figure 3.42. Effect of inhibition of the NF-κB signalling pathway on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 2 h with PDTC, the NF-κB inhibitor (10 μM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding -PDTC treatment.

3.5.3. AP-1 Signaling Pathway Modulates the Expression of *Nqo1* Gene by metals

To investigate the role of the AP-1 signaling pathway in the modulation of the *Nqo1* gene by metals, cells were pre-treated for 2 h with a JNK inhibitor, SP600125 (10 μM), an ERK inhibitor, U0126 (10 μM), and a p38 MAPK inhibitor, SP203580 (10 μM) prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), and TCDD (1 nM) for indicated time periods. If these MAPK activities are critical, their inhibition would prevent metal-mediated effects on *Nqo1* gene expression.

3.5.3.1. Inhibition of the JNK Signaling Pathway Suppresses the Metal-Mediated Induction of Nqo1 Gene Expression

Treatment of Hepa 1c1c7 cells with SP600125 (10 μM), a concentration known to inhibit the JNK signaling cascade and subsequently AP-1 activity (Chen et al., 2006), completely suppressed both constitutive and metal-mediated induction of Nqo1 at mRNA levels and activity levels in a manner similar to what was observed with TCDD treatment (Fig. 3.43A and B). The maximum inhibitory effects of SP600125 were observed with Cu^{2+}-mediated induction of Nqo1 activity (Fig. 3.43B).

3.5.3.2. Inhibition of the ERK Signaling Pathway Modulates the Metal-mediated Induction of Nqo1 Gene Expression

The ERK signaling pathway inhibitor, U0126 (10 μM), significantly increased constitutive Nqo1 mRNA and enzyme activity levels by approximately 100% and 27%, respectively (Fig. 3.44). Co-treatment with U0126 and metals further potentiated the induction of Nqo1 mRNA levels by all three metals (Fig. 3.44A).

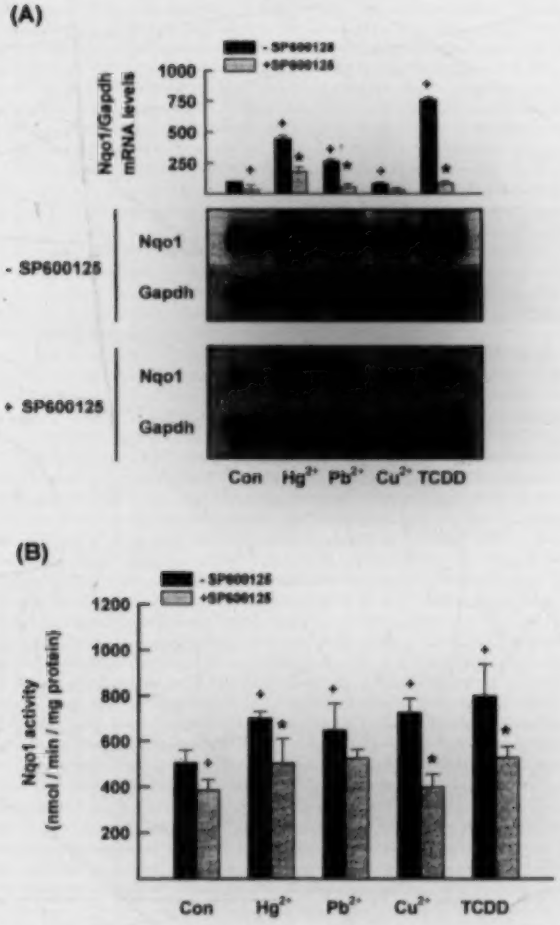

Figure. 3.43. Effect of inhibition of the JNK signalling pathway on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 2 h with SP600125, a JNK inhibitor (10 μM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding –SP600125 treatment.

The maximum induction was observed with Cu^{2+} (75%), followed by Hg^{2+} and Pb^{2+} by approximately 27%. However, U0126 did not significantly alter the TCDD-mediated effect at Nqo1 mRNA level (Fig. 3.44A). On the other hand, co-treatment with U0126 and metals significantly potentiated the induction of Nqo1 at activity levels by Hg^{2+} only, but did not alter other metal- or TCDD-mediated effects (Fig. 3.44B).

3.5.3.3. Inhibition of p38 the MAPK Signaling Pathway Modulates the Metal-Mediated Induction of Nqo1 Gene Expression

Although treatment of Hepa 1c1c7 cells with SB203580 (10 μM) significantly induced constitutive Nqo1 mRNA and activity levels, it significantly decreased the TCDD-mediated effect (Fig. 3.45A and B). Co-treatment with SB203580 and metals significantly potentiated only Pb^{2+}- and Cu^{2+}-mediated effects at the mRNA levels (Fig. 3.45A), but did not significantly alter metal-mediated effects at the activity levels (Fig. 3.45B).

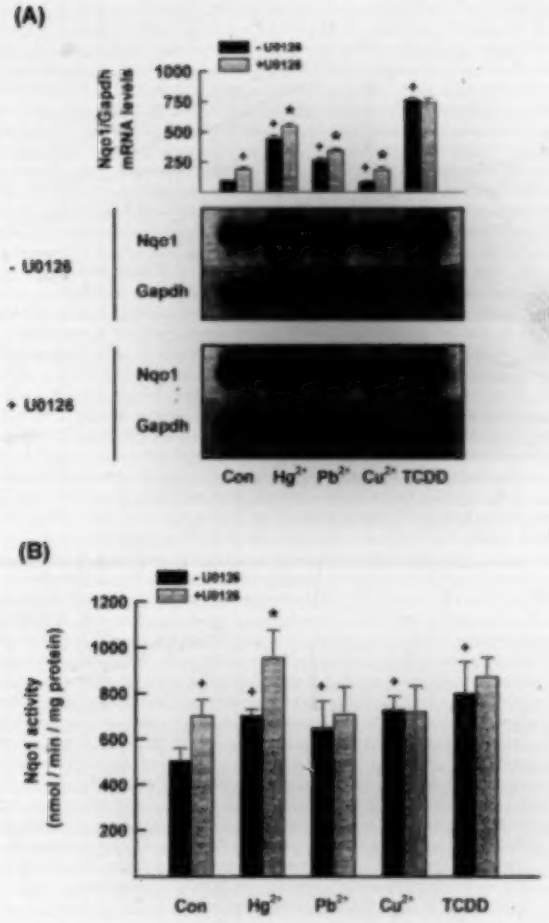

Figure 3.44. Effect of inhibition of the ERK signalling pathway on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 2 h with U0126, an ERK inhibitor (10 μM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. . Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding –U0126 treatment.

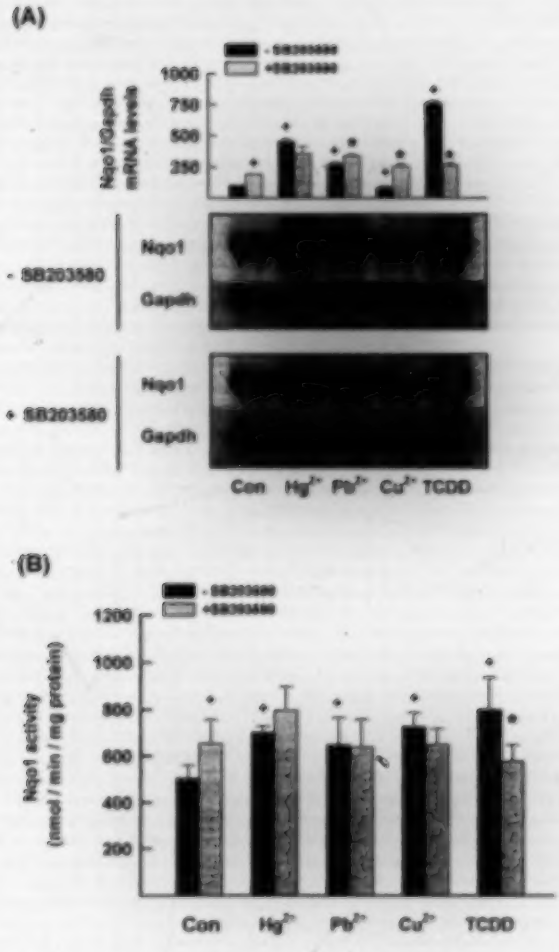

Figure 3.45. Effect of inhibition of the p38 MAPK signalling pathway on the induction of Nqo1 mRNA (A) and activity (B) levels by metals. (A) Hepa 1c1c7 cells were pre-treated for 2 h with SB203580, a p38 MAPK inhibitor (10 μM), prior to the addition of Hg^{2+} (5 μM), Pb^{2+} (25 μM), Cu^{2+} (10 μM), or TCDD (1 nM) for an additional 6 h. . Nqo1 mRNA normalized to Gapdh mRNA was determined by Northern blot analysis. Only one of three representative experiments is shown. (B) Nqo1 enzyme activity (mean ± SEM, n=6) was determined spectrophotometrically using DCPIP as a substrate. $^{+}$, $p < 0.05$ compared with control (Con); and *, $p < 0.05$ compared with corresponding –SB203580 treatment.

CHAPTER 4- DISCUSSION

4.1 GENERAL DISCUSSION

Both simultaneous and sequential exposure to metals, such as Hg^{2+}, Pb^{2+} or Cu^{2+}, and AhR ligands is a common environmental problem with biological consequences. Yet there have been relatively few studies of the combined effects of metals and AhR ligands on AhR-regulated genes. The results of this study were important as they showed the potential interactions between metals and PAHs on the regulation of AhR-regulated genes, and hence the toxicity and carcinogenicity of PAHs. In addition, the current study explores the molecular mechanisms involved.

Trace metals are highly toxic non-essential elements in that they are neither created nor biodegradable (Barbier et al., 2005). Long-term human exposure to metals from numerous sources, including contaminated air, water, soil, and food due to industrial activities, is a common environmental problem with persistent biological consequences. Co-contamination of metals with several environmental contaminants such as PAHs, and their effects on the mutagenicity and carcinogenicity of these contaminants, make them ranked highly among the top 20 hazardous substances (ATSDR, 2005; CEPA, 2006). Yet, the effects of metals on the modulation of AhR-regulated genes and the molecular mechanisms involved remained undetermined.

The concentrations of metals used in the current study were chosen after determining the ability of a wide range of concentrations to modulate the expression of AhR-regulated genes without significantly affecting Hepa 1c1c7 cell viability (Fig. 3.1). In addition, the estimated human plasma and tissue concentrations of these metals in individuals without

known exposure have been reported in low μM range (Gerhardsson et al., 1988; Tezel et al., 2001) and within the range that might be expected to be encountered in the environment (Vakharia et al., 2001b; Vakharia et al., 2001a; ATSDR, 2005; CEPA, 2006). Taken together, chronic human exposure to these toxic metals, their longer half lives, and the high possibility of accumulations in the body tissues (Barbier et al., 2005), make the concentrations used in the present study highly relevant to the corresponding human plasma and tissue levels and related to calculated exposure levels in the environment (ATSDR, 2005; CEPA, 2006).

4.1.1 Hg^{2+}, Pb^{2+}, and Cu^{2+} Differentially Modulate the Expression of AhR-Regulated Genes

Initially, we have investigated the potential capacity of three prominent environmental metal contaminants, Hg^{2+}, Pb^{2+} and Cu^{2+}, to modulate the induction of *Cyp1a1*, *Nqo1*, and *Gsta1* genes by four potential AhR ligands, TCDD, 3MC, βNF, and BaP at the activity and mRNA levels.

Our results showed that the AhR-regulated genes tested, *Cyp1a1*, *Nqo1*, and *Gsta1*, exhibited variable sensitivity to the induction by AhR ligands. In the present study, we showed that Cyp1a1 was the most sensitive, followed by Nqo1, whereas Gsta1 was the least sensitive, suggesting that Cyp1a1 is a much more sensitive biomarker than Nqo1 or Gsta1 assays for AhR ligands. The potency of AhR ligands to induce Cyp1a1, at the concentrations tested, was in the order βNF>3MC>TCDD>BaP (Figs. 3.2 and 3.7-3.10), whereas the order of potency of induction of Nqo1 and Gsta1 by AhR ligands was

βNF>3MC>BaP>TCDD; and βNF>BaP>3MC>TCDD, respectively (Figs. 3.3, 3.4, 3.7-3.10). On the other hand, metals in the absence of AhR ligands did not significantly alter the constitutive Cyp1a1 at the activity level in WT cells. Interestingly, all three metals significantly increased Cyp1a1 mRNA levels in a concentration-dependent manner, with the only exception that Cu^{2+} at the highest concentration tested did not induce Cyp1a1 expression (Fig. 3.6).

Regulation of the *CYP1A1* gene has been shown to involve the activation of the AhR-dependent pathway by direct binding of AhR ligands to the receptor, AhR (Whitlock, 1999). Interestingly, metals do not exhibit any structural properties and similarities to classical AhR ligands, suggesting that they could be novel non-classical modulators of AhR which are able to induce *Cyp1a1* gene expression but do not appear to bind directly to AhR. An initial study by Kapitulnik and Gonzalez (1993) showed that rats with congenital jaundice, manifested by severe hyperbilirubinemia, exhibited an endogenous activation of *CYP1A1/1A2* gene expression (Kapitulnik and Gonzalez, 1993). These observations have been interpreted as indirect evidence for the existence of endogenous AhR ligand(s), for example bilirubin (Denison and Nagy, 2003). In addition, it was proposed that these non-classical AhR ligands could be converted by themselves into classical ligands or activate some other pathways that result in AhR activation (Denison and Nagy, 2003). In this respect, all metals tested in the current study induced HO-1 mRNA expression (Fig. 3.6), a rate limiting enzyme in the degradation of heme to bilirubin (Sinal and Bend, 1997), resulting in increased bilirubin formation which has

been previously shown to induce Cyp1a1 expression in Hepa 1c1c7 cells through an AhR-dependent pathway (Sinal and Bend, 1997).

The ability of AhR ligands to induce Cyp1a1 was significantly inhibited at the activity level by all metals tested (Fig. 3.2). Cu^{2+}, for example, exhibited the highest inhibitory effect on all AhR ligands-mediated induction of Cyp1a1 activity, followed by Hg^{2+} and Pb^{2+}. Interestingly, we have observed that the increase in HO-1 mRNA level coincided with the decrease in Cyp1a1 and the increase in Nqo1 and Gsta1 catalytic activities. However a firm link between HO-1 induction and the modulation in phase I and phase II enzyme activities has not been established yet.

The capability of Hg^{2+} (Stohs and Bagchi, 1995; Kaliman et al., 2001), Pb^{2+} (Stohs and Bagchi, 1995), and Cu^{2+} (Ossola et al., 1997), to induce HO-1 has been reported previously. These studies suggested that metal-mediated oxidative stress, including the production of ROS (Stohs and Bagchi, 1995; Kaliman et al., 2001) and lipid peroxidation (Stohs and Bagchi, 1995; Ossola et al., 1997), which subsequently activate HO-1 transcription via the ARE and nuclear factor-I (NFI) is a possible mechanism for the induction of HO-1 by metals (Inamdar et al., 1996). Thus, the differential sensitivity of each AhR ligand to metals could be an indicator to how much oxidative stress and HO-1 is activated and to what extent they could counteract the potency of induction by AhR ligands. Alternatively, it has been reported that metals inhibit δ-aminolevulinic acid dehydratase, an important enzyme in the heme synthesis pathway, via binding of the metals, such as Pb^{2+}, to thiol groups of allosteric sites to provoke allosteric transitions to

an inactive form of the enzyme leading to a decrease in the heme synthesis (Bernard and Lauwerys, 1987). Considering that increased HO-1 mRNA expression would increase heme degradation and since heme is the prosthetic group of CYP, we postulated that decreased Cyp1a1 activity by metals could be attributed to a decrease in heme availability.

The results of the current study showed that the decrease in the AhR ligand-mediated induction of Cyp1a1 activity by Hg^{2+} was associated with an increase in Cyp1a1 mRNA, suggesting a post-transcriptional mechanism might be involved in the inhibition of Cyp1a1 activity (Figs. 3.2 and 3.7-3.10). On the other hand, Pb^{2+} post-translationally inhibited the induction of Cyp1a1 activity, which was associated with no significant changes in Cyp1a1 mRNA levels. In contrast, Cu^{2+}, inhibited the induction of Cyp1a1 by all AhR ligands at the activity, mRNA, suggesting a pre-transcriptional mechanism (Figs. 3.2 and 3.7-3.10). Our results were in agreement with previous studies which showed an increased mRNA expression and decreased activity of Cyp1a1 in mice treated with toluene diisocyanate, a non-AhR ligand industrial chemical (Haag et al., 2002). Similarly, BaP-induced CYP1A1 activity in HepG2 cells was inhibited by ellipticine, a CYP1A1 inhibitor, whereas CYP1A1 mRNA was increased (Morel et al., 1999). Decreases in the activity and increases in the mRNA expression could be attributed to two main postulated mechanisms: the first mechanism is through the activation of endogenous ligands which activate the AhR leading to the induction of *CYP1A1* gene transcription (Chang and Puga, 1998; Nebert et al., 2000). The second mechanism, which is less well supported by experimental studies, is that the induction of CYP1A1 enzyme activates an endogenous

substrate to become a repressor; therefore, inhibition of CYP1A1 activity by metals would lead to inhibition of the repressor action and, hence, activation of gene transcription (Nebert et al., 2000). These observations indicate that the increased gene expression could result from the decreased activity, suggesting a negative feedback autoregulatory loop (Morel et al., 1999).

In the current study all metals alone induced Nqo1 and Gsta1 at the activity and mRNA levels (Figs. 3.3, 3.4, and 3.6-3.10). The induction is more potent in WT than in AhR-deficient C12 cells (Fig. 3.5). In addition, Nqo1 was more sensitive to metal effects than Gsta1; however, Cu^{2+} exhibited the most potent inducing effect on the activities of both genes, suggesting that these metals are potent inducers of phase II enzymes. On the other hand, Cu^{2+} significantly increased the induction of Nqo1 at the activity level but decreased its mRNA expression. Although the mechanism could not be interpreted from our results, it could be attributed to an increase in the post-transcriptional degradation of mRNA.

Co-administration of metals with AhR ligands exhibited variable effects on Gsta1. For example, at the activity level, only Cu^{2+} potentiated the induction of Gsta1 activity by all AhR ligands tested (Fig. 3.5), whereas at the mRNA level, Hg^{2+} and Pb^{2+} increased, but Cu^{2+} decreased, Gsta1 mRNA expression (Figs. 3.6-3.10). In AhR-deficient C12 cells, Hg^{2+} and Pb^{2+}, but not Cu^{2+}, significantly increased Nqo1 activity, suggesting that an AhR-independent mechanism, such as an ARE-mediated mechanism, is involved in the regulation of Nqo1 and Gsta1 (Paulson, 1989; Jaiswal, 2000).

The activation of ARE-driven transcription by metals has been reported previously (Kong et al., 2001). One of the suggested mechanisms by which metals activate the ARE-mediated pathway is through activation of MAPKs and PKs via the production of ROS. Once activated, MAPKs and PKs will then phosphorylate the Nrf2/Keap1 complex, resulting in release of Nrf2, allowing it to translocate to the nucleus to transactivate ARE on the promoter region of *NQO1* and *GSTA1* genes (Kong et al., 2001; Ma et al., 2004). Another postulated mechanism is through activation of redox-sensitive transcription factors such as NF-κB, which results in an increase in gene expression (Chen et al., 2001).

We conclude that metals differentially modulate the constitutive and inducible expression of AhR-regulated genes. The current study suggests that chronic exposure to a metals/AhR ligand mixture would be less effective in inducing AhR-mediated carcinogenicity than exposure to AhR ligands alone through reducing the Cyp1a1 activity, a carcinogen-activating enzyme, and increasing the activities of Nqo1 and Gsta1, detoxifying genes.

4.1.2. Hg^{2+}, Pb^{2+}, and Cu^{2+} Modulate the Expression of *Cyp1a1* Gene through Transcriptional and Post-Translational Mechanisms

To further explore the molecular mechanisms involved in the modulation of Cyp1a1 by metals, we examined the role of transcriptional and/or post-transcriptional mechanisms. For this purpose, a series of experiments were carried out using a single but different

concentration for each metal; these were 5, 25, and 10 μM for Hg^{2+}, Pb^{2+}, and Cu^{2+}, respectively. In addition, TCDD, 1 nM, was used to examine the mechanisms at the inducible levels.

Initially, we have found that all tested metals significantly increased the basal Cyp1a1 mRNA levels in a time-dependent manner (Fig. 3.11). Interestingly, the induction pattern of Cyp1a1 mRNA by metals was similar to those obtained with TCDD, in which metals caused a rapid increase in Cyp1a1 mRNA, which was evident after 3 h of treatment, reaching steady-state after 12 h. Although metal-mediated induction of Cyp1a1 mRNA was 10-fold lower than that induced by TCDD, the level of induction was 15-fold higher than control values (Fig. 3.11). On the other hand, the TCDD-mediated induction of Cyp1a1 mRNA was further increased by Hg^{2+} and Pb^{2+}, but was decreased by Cu^{2+} co-treatment. Therefore, the alteration in Cyp1a1 mRNA steady-state levels in response to metals could be attributed to transcriptional and/or post-transcriptional mechanisms.

The transcriptional regulation of *Cyp1a1* gene expression by metals was demonstrated through different approaches. First, the inhibition of the RNA transcription, using Act-D, completely abolished the induction of Cyp1a1 mRNA in response to metals, implying that metals increase the *de novo* Cyp1a1 RNA synthesis, in a manner similar to that obtained with TCDD (Fig. 3.13). Second, the co-administration of metals with CHX, a protein translation inhibitor, and/or MG-132, a 26S proteasome inhibitor, superinduced the Cyp1a1 mRNA in response to metals (Figs. 3.14 and 3.15). Superinduction of Cyp1a1 mRNA by CHX or MG-132 has been previously reported in Hepa 1c1c7 (Sindhu and

Kikkawa, 1999; Ma et al., 2000; Gharavi and El-Kadi, 2005) and in other cell lines (Daujat et al., 1991; Lamb and Franklin, 2002; Joiakim et al., 2004). Recent studies have demonstrated that the superinduction of the *Cyp1a1* gene by CHX or MG-132 is a transcriptional mechanism and reflects a change in the synthesis, rather than stabilization, of Cyp1a1 mRNA (Ma and Baldwin, 2000; Ma and Baldwin, 2002; Joiakim et al., 2004).

Interestingly, it has been previously reported that superinduction of the transcription of AhR-regulated genes by CHX or MG-132 is a gene-specific effect. For example, it has been shown that *Cyp1a1* and TCDD-inducible poly(ADP-ribose) polymerase (TiPARP), which are regulated by AhR, are superinduced by CHX or MG-132 (Ma, 2002), whereas the induction of phase II AhR-regulated genes, *Nqo1* and *Gsta1*, by TCDD requires both AhR and Nrf2 proteins (Ma et al., 2004). This is because Nrf2 is a labile protein, and therefore, the induction is susceptible to CHX but not MG-132.

Several proposed mechanisms of Cyp1a1 superinduction have been reported. The most acceptable mechanism suggests the presence of a labile transcriptional protein that negatively regulates the expression of *Cyp1a1*. This is supported by observations that inhibition of protein synthesis, using CHX, decreases the concentration of repressor proteins, such as IκBa (Ma et al., 2000), resulting in an increase in the rate of *Cyp1a1* transcription (Israel et al., 1985; Lusska et al., 1992; Lamb and Franklin, 2002). Moreover, it has been hypothesized that inhibition of the turnover of AhR protein by a protein synthesis inhibitor (Ma et al., 2000; Ma and Baldwin, 2002) or proteasome inhibitor (Ma and Baldwin, 2000; Pollenz, 2002) accounts for the superinduction of

Cyp1a1, based on the fact that AhR activation is an integral step for the induction of *Cyp1a1* gene expression (Ma et al., 1995). Taken together, these results suggest that the superinduction of Cyp1a1 in response to metals in the presence of CHX or MG-132 is both a transcriptional and an AhR-dependent pathway, in a manner similar to that obtained with TCDD (Sindhu and Kikkawa, 1999; Ma and Baldwin, 2002).

The direct evidence for the involvement of the AhR in the transcriptional regulation of *Cyp1a1* by metals comes from the results of the EMSA. The presence of a nuclear AhR complex is dependent on ligand binding to the cytosolic receptor, nuclear translocation of the liganded AhR, its heterodimerization with ARNT, and subsequent specific and high-affinity DNA binding (Phelan et al., 1998). Our results not only suggest that metals can induce AhR transformation (*in vitro*), nuclear accumulation (*in vivo*), and DNA binding, similar to that which is observed with classical AhR ligands such as TCDD (Fig. 3.16), but also support a role for the AhR in the induction of *Cyp1a1* by metals. Surprisingly, unlike Hg^{2+} and Pb^{2+}, Cu^{2+} fails to induce the nuclear AhR/ARNT/XRE complex formation. At present, we have no mechanistic explanation for the differential effects of Cu^{2+} *in vitro* and in intact cells. However, these results indicate the involvement of different molecular processes.

The mechanism by which metals directly activate the *in vitro* AhR translocation is still unknown. However, several mechanisms of activation of the AhR by nonclassical ligands have been proposed. In this regard, it has recently been demonstrated that the divalent metal arsenite induced AhR nuclear translocation and binding to the *Cyp1a1* gene

promoter in Hepa 1c1c7 cells, possibly by disrupting the molecular interaction between the AhR and its associated protein, HSP90 (Kann et al., 2005). Based on these observations, we postulate that metals may bind to the AhR-associated proteins, or to a non-TCDD binding site on the receptor, resulting in AhR conformational changes which lead to activation of the receptor and its translocation to the nucleus (Werlinder et al., 2001).

To test the hypothesis that metals may modulate Cyp1a1 mRNA at the post-transcriptional level, we assessed the turnover rate of Cyp1a1 mRNA using an Act-D chase experiment. Our results showed that the Cyp1a1 mRNA induced by TCDD is short-lived, with an estimated half-life of 3.8 h (Fig. 3.17). These results are in agreement with previous reports that estimated the half-life of Cyp1a1 mRNA induced by TCDD in Hepa 1c1c7 cells ranges from 3–4.5 h (Miller et al., 1983; Chen et al., 1995). Previous studies have reported that the half-life for Cyp1a1 mRNA induced by a different AhR ligands such as dimethybenzanthracene in MCF7 cells was about 7 h (Ciolino and Yeh, 1999), whereas in human hepatoma HepG2 cells the half-life was about 2.5 h (Lekas et al., 2000). The differences in the half-lives could be attributed to different cell lines, Cyp1a1 inducers, and/or the type of RNA synthesis inhibitor used. In our study, the Cyp1a1 mRNA half-life in Hepa 1c1c7 cells treated with metals was not statistically different from those of TCDD-induced Cyp1a1 mRNA, indicating that metals did not alter the stability of Cyp1a1 mRNA. In agreement with our results, but with a different metal, it has been recently reported that arsenite did not affect the Cyp1a1 mRNA stability in HepG2 cells (Bessette et al., 2005).

The inhibitory effects of metals on the induction of Cyp1a1 activity by TCDD suggest a post-translational mechanism, though the post-translational regulation of *Cyp1a1* is not well studied. The CHX-chase experiment showed that Cyp1a1 protein is short-lived, with a half-life of about 2.7 h (Fig. 3.18). Similarly, in V79 cells, the half-life of Cyp1a1 protein was 2.8 h (Werlinder et al., 2001). To our knowledge, this is the first demonstration that all three metals significantly decreased the degradation rate of Cyp1a1 protein (Fig. 3.18), providing the first evidence that metals are capable of regulating *Cyp1a1* gene expression through a post-translational mechanism.

Interestingly, the stabilization of Cyp1a1 protein by metals (Fig. 3.18) was in contrast with the inhibitory effects of metals on the induction of Cyp1a1 activity mediated by TCDD (Figs. 3.2 and 3.19). Although it is unclear how metals decreased the induction of Cyp1a1 activity, it has been previously demonstrated that a decrease in heme availability, the prosthetic group of CYP, causes a reduction in the Cyp1a1 activity (Meyer et al., 2002). This is supported by our current observations that metals induced HO-1 mRNA expression and decreased cellular heme content. Another potential mediator that may be involved in the inhibition of TCDD-mediated induction of Cyp1a1 activity by metals is ROS. We have recently demonstrated that increased ROS production by the AhR ligands, BaP, 3MC, and βNF, at high concentrations was accompanied by a decrease in the Cyp1a1 catalytic activity, but not the mRNA or protein expression levels, which were significantly induced in a concentration-dependent manner (Elbekai et al., 2004). The AhR-mediated decrease in Cyp1a1 activity was reversed by the antioxidant *N*-

acetylcysteine, suggesting a direct involvement of ROS in the inhibition of Cyp1a1 activity.

In conclusion, data presented here clearly demonstrate that Hg^{2+}, Pb^{2+} and Cu^{2+} can directly activate the AhR and modulate *Cyp1a1* gene expression at both transcriptional and post-translational levels. Furthermore, the inhibitory effects of metals on the TCDD-inducible Cyp1a1 activity are attributed, at least in part, to decreased heme availability.

4.1.3. Hg^{2+}, Pb^{2+}, and Cu^{2+} Modulate the Expression of *Nqo1 and Gsta1* Genes through AhR/XRE- and Nrf2/ARE-Dependent Transcriptional Mechanisms

The present study demonstrates several important differences between metals and TCDD with regard to their effects on the kinetics of Nqo1 and Gsta1 mRNA and activity levels. Hg^{2+} and Pb^{2+} show a rapid onset of induction compared with TCDD. However, TCDD shows a longer duration of induction. Hg^{2+}- or Pb^{2+}-mediated induction of Nqo1 and Gsta1 mRNAs returned to the basal level at 24 and 12 h, respectively. In contrast, TCDD-mediated induction remained elevated for at least 24 h. Furthermore, the magnitude of the Hg^{2+}-mediated induction of Nqo1 mRNA was similar to that observed with TCDD, whereas the induction of Gsta1 mRNA by Hg^{2+} was 2-fold higher than that of TCDD (Figs. 3.20 and 3.21).

Initially, we questioned whether the induction of Nqo1 and Gsta1 mRNAs by the three metals is regulated by a transcriptional mechanism in which metals increase *de novo* RNA synthesis and/or by a post-transcriptional stabilization of the mRNA. Thus, a series of

experiments was carried out to determine which molecular mechanisms these metals target.

The transcriptional regulation of *Nqo1* and *Gsta1* genes by metals was demonstrated by different approaches. First, we have shown that the RNA synthesis inhibitor Act-D abolished the metal-mediated induction of Nqo1 and Gsta1 mRNAs (Fig. 3.24). These results imply that metals increase *de novo* synthesis of Nqo1 and Gsta1 RNAs in a manner similar to that observed with TCDD, which is known to induce these genes at the transcriptional level through the XRE-ARE pathway (Radjendirane and Jaiswal, 1999; Ma et al., 2004; Marchand et al., 2004; Miao et al., 2005). Second, the protein synthesis inhibitor CHX significantly inhibited the metal-mediated induction of Nqo1 and Gsta1 mRNAs through a transcriptional mechanism, in which CHX inhibited the newly synthesized Nqo1 and Gsta1 mRNAs but did not affect the existing mRNAs Figs. 3.25 and 3.26). Our results are in agreement with previous published studies in different cells lines that demonstrated that CHX inhibited the induction of Nqo1 and Gsta1 mRNAs through a transcriptional mechanism (Eickelmann et al., 1995; Lamb and Franklin, 2002; Ma et al., 2004). Furthermore, the inhibition of metal-mediated induction of Nqo1 and Gsta1 mRNAs by CHX was not due to a decrease in cell viability or general inhibitory effect on gene transcriptions, since the same concentration of CHX superinduced the metal-mediated induction of Cyp1a1 mRNA (Fig. 3.14).

The ability of metals to induce *Nqo1* and *Gsta1* genes in both WT and C12 Hepa 1c1c7 cells suggested the involvement of XRE- and ARE-dependent mechanisms (Fig. 3.5). In

this regard, we demonstrated that the metals directly activate the AhR and hence the XRE-dependent pathway (Fig. 3.16). On the other hand, it has been shown that ARE-dependent transcriptional activation of *Nqo1* and *Gsta1* genes requires the activation of a CHX-sensitive labile protein under oxidative stress (Rushmore and Kong, 2002; Ma et al., 2004; Miao et al., 2005; Xu et al., 2005). Itoh et al. have identified a labile protein, namely Nrf2, that binds to ARE and subsequently activates *Nqo1* and *Gsta1* transcription (Itoh et al., 1997). Nrf2 is a basic leucine zipper transcriptional protein that is degraded rapidly by the proteasome pathway (Chen and Kunsch, 2004; Jaiswal, 2004; Ma et al., 2004). Noda and coworkers showed that only inducible, but not constitutive, *Nqo1* and *Gstp* gene expression was abolished in Nrf2-null mice. Conversely, in AhR- and Nrf2-double knockout mice, both constitutive and inducible expression of *Nqo1* and *Gstp* genes was completely abolished (Noda et al., 2003). Similar results were observed in Nrf2-knockout mice embryonic fibroblast cells (Hayes et al., 2000; Jaiswal, 2004; Ma et al., 2004; McWalter et al., 2004; Nioi and Hayes, 2004). These results clearly indicate that both AhR- and Nrf2-mediated pathways play an integral role in the regulation of *Nqo1* and *Gsta1* genes. More recently, it has been shown that a cross-talk exists between AhR-XRE and Nrf2-ARE, in which Nrf2 gene expression is directly regulated through AhR activation (Ma et al., 2004; Nioi and Hayes, 2004; Miao et al., 2005). Moreover, Marchand et al. demonstrated that the *NQO1* gene expression can be controlled by activity of CYP1A1, a phase I enzyme that is regulated mainly through an AhR-dependent pathway, through an oxidative stress-mediated pathway (Radjendirane and Jaiswal, 1999; Marchand et al., 2004).

The notion that a CHX-sensitive labile protein, Nrf2, mediates the regulation of *Nqo1* and *Gsta1* genes by metals is supported by several observations. Initially, the protein synthesis inhibitor, CHX, at a concentration shown to inhibit Nrf2 protein synthesis (Ma et al., 2004), blocked the constitutive and the metal-mediated induction of Nqo1 and Gsta1 mRNAs (Fig. 3.26). In addition, the inhibition of proteasome-dependent degradation of Nrf2 by MG-132, a 26S proteasome inhibitor, in the presence of CHX plus metals reversed the inhibitory effects of CHX on the induction of Nqo1 and Gsta1 mRNAs by metals (Fig. 3.26 and 3.27A and B). Most importantly, we have shown that metals not only require Nrf2 for the induction of these genes but also increase the Nrf2 protein level (Fig. 3.27C). Thus, it is possible that the increases in Nqo1 and Gsta1 mRNAs observed with Hg^{2+} and Pb^{2+} could be attributed to the ability of these metals to decrease the turnover rate of Nrf2 protein.

In agreement with previous reports (Kwak et al., 2002; Ma et al., 2004), we demonstrated that blocking the Nrf2 degradation alone using MG-132 is not sufficient to induce the expression of basal Nqo1 or Gsta1 mRNA, although it increases total cellular Nrf2 level. This effect could be attributed to the fact that MG-132 increases the level of total cellular Nrf2 but does not increase the nuclear level of Nrf2, which will be sequestered in the cytoplasm. Therefore, it has been hypothesized that a Nrf2 inducer is required to enhance the nuclear translocation of Nrf2 to transactivate the ARE-mediated genes. Moreover, the discrepancy in the effects of CHX in the presence and absence of MG-132 suggests that CHX does not affect the level of existing Nrf2 proteins; rather, it inhibits its *de novo*

protein synthesis, which is required by metals for the induction of *Nqo1* and *Gsta1* genes (Kwak et al., 2002).

It is still unclear how metals activate the Nrf2-ARE-mediated transcriptional induction of *Nqo1* and *Gsta1* genes. However, it has been postulated that phosphorylation signaling pathways may contribute to the induction of *Nqo1* and *Gsta1* genes by metals, through MAPKs-mediated phosphorylation of Nrf2/Keap1 complex in the cytoplasm (Radjendirane and Jaiswal, 1999; Jaiswal, 2004) resulting in activation of the Nrf2-ARE pathway (Huang et al., 2002; Jonak et al., 2004; Kim et al., 2005).

To test the hypothesis that metals may modulate *Nqo1* or *Gsta1* genes at the post-transcriptional and/or post-translational levels, we assessed the turnover rates of their mRNA and protein. Our results showed that the constitutive and inducible expression of Nqo1 and Gsta1 transcripts are long-lived mRNAs with estimated half-lives of approximately 17 and 11 h, respectively (Figs. 3.28 and 3.29). These results are in agreement with previous studies that reported a half-life of >15 h for Nqo1 mRNA in Hepa 1c1c7 cells (Ma et al., 2004) and 14.5 h for Gsta1 mRNA in human HepG2 cells (Eickelmann et al., 1995). However, Nqo1 and Gsta1 mRNA half-lives in Hepa 1c1c7 cells treated with metals were not statistically different from their corresponding control mRNA, indicating that metals do not regulate the *Nqo1* and *Gsta1* genes at the post-transcriptional level (Figs. 3.28 and 3.29). Furthermore, our results showed that constitutive and inducible Nqo1 and Gsta1 are long-lived proteins with estimated half-lives >24 h (Figs. 3.30 and 3.31), which is in agreement with a previously published

study (Siegel et al., 2001). In addition, the stability of both proteins was not significantly altered by metals up to 24 h, indicating the lack of post-translational regulation of the *Nqo1* and *Gsta1* genes by metals (Figs. 3.30 and 3.31).

In conclusion, we have provided strong evidence that metals regulate the expression of *Nqo1* and *Gsta1* genes through AhR-XRE- and Nrf2-ARE-dependent transcriptional mechanisms. Although metals are considered toxic and ranked highly as the most hazardous substances in the environment (ATSDR, 2005; CEPA, 2006), we have demonstrated that metals may have cytoprotective properties by increasing the expression of *Nqo1* and *Gsta1* genes, which are enzymes considered to be protective against carcinogenesis.

4.1.4. NF-κB and AP-1 Signaling Pathways Modulate the Expression of the *Cyp1a1* Gene by Hg^{2+}, Pb^{2+}, and Cu^{2+}

Several studies have shown that higher intracellular concentrations of metals are toxic through various pathways, of which an imbalance of redox in favour of oxidation is one of these mechanisms (Valko et al., 2005). Generation of ROS by metals, which induces oxidative stress, causes cell membrane damage, enzyme inactivation, altered gene expression, and finally apoptosis (Ercal et al., 2001; Valko et al., 2005). Several studies have reported that altering the cellular redox status acts as a cellular messenger to modulate enzyme activity, transcriptional factors, and MAPK signaling cascades (Hancock et al., 2001; Leonard et al., 2004). Therefore, the possibility that redox-sensitive transcription factors, such as NF-κB and AP-1 may directly or indirectly be

involved in the modulation of AhR-regulated genes by metals could not be ruled out. Therefore we examined the role of NF-κB and AP-1 signaling pathways in the modulation of the *Cyp1a1* gene by Hg^{2+}, Pb^{2+}, and Cu^{2+}.

Initially, we have tested the capacity of three metals to alter the redox status of Hepa 1c1c7 cells. We first demonstrated that Hg^{2+}, Pb^{2+}, and Cu^{2+} significantly increased ROS production and HO-1 mRNA expression in a concentration-dependent manner (Fig. 3.32). In this context, we previously showed that Hg^{2+}, Pb^{2+}, and Cu^{2+} increased ROS production in WT but not in AhR-deficient C12 Hepa 1c1c7 cells, suggesting an AhR-dependent mechanism (Korashy and El-Kadi, 2006a). The ability of these metals to induce oxidative stress and ROS formation has been previously reported (Mattie and Freedman, 2004; Valko et al., 2006).

One of the mechanisms of metal-mediated oxidative stress is through the ROS generation via Fenton- and Haber–Weiss-type reactions. In addition, the ability of metals to covalently bind to protein sulfhydryl group which leads to GSH depletion and subsequent inhibition of cellular antioxidant enzymes, such as GSH peroxidase (GP), catalse, and superoxide dismutase, is considered the most important mechanism (Ercal et al., 2001; Mattie and Freedman, 2004). It has been shown that rat hepatic catalase and GP activities were significantly decreased 5 h after Cu^{2+} injection, which was accompanied with increases in ROS formation and HO-1 activity. Furthermore, Hg^{2+} induces hydrogen peroxide formation and lipid peroxidation in rat kidney mitochondria by blocking

electron transport at the ubiquinone–cytochrome *b5* step (Ercal et al., 2001; Mattie and Freedman, 2004).

GSH is an important cellular antioxidant that stabilizes metal in its neutralized state, preventing redox cycling and free radical generation (Oliveira et al., 2004). Therefore, we examined whether or not GSH depletion would potentiate the metal-mediated effect. In this regard, we showed here that cellular GSH depletion by blocking its synthesis potentiated metal-induced oxidative stress which was accompanied with a further increase in metal-mediated effects on Cyp1a1 mRNA and activity levels (Fig. 3.33). BSO significantly potentiated the inhibitory effects of the metals on Cyp1a1 activity (Fig. 3.33C). Our results are in agreement with previous reports which showed that GSH protected against Hg^{2+}- and Cu^{2+}-mediated inhibition of hepatic CYP1A1 activity in a sea bass *Dicentrarchus labrax L.* (Otto et al., 1999; Oliveira et al., 2004). Taken together, these results strongly suggest that the modulation of the *Cyp1a1* gene by metals is controlled by oxidative stress-dependent mechanisms.

Increased cellular oxidative stress has been shown to function as a second messenger in cellular signaling pathways to activate various redox-sensitive transcription factors, particularly NF-κB and AP-1 (Leonard et al., 2004). In this regard, we demonstrated here that metal-induced oxidative stress was able to trigger activation of NF-κB and AP-1 binding activity in a metal-dependent manner (Fig. 3.34). The differences in extent of NF-κB and AP-1 activation by Hg^{2+}, Pb^{2+}, and Cu^{2+} could be attributed to the differential capacity of these metals to alter the redox status of the cells (Fig. 3.32).

The involvement of NF-κB was assessed by determining the effect of the NF-κB activator and inhibitor, PDTC and PMA, respectively. Our results clearly demonstrate that activation of NF-κB signaling pathways may negatively regulates the induction of the *Cyp1a1* gene in response to metals (Fig. 3.35 and 3.36). This conclusion was derived from the observation that PMA inhibited, whereas PDTC potentiated, the metals-mediated effects on *Cyp1a1* and *HO-1* genes.

The inhibitory interaction between the AhR and NF-κB signaling pathways has been reported previously (Tian et al., 1999; Kim et al., 2000; Tian et al., 2002). Although the exact mechanisms by which NF-κB modulates *Cyp1a1* gene expression by metals were not investigated in the current study, we postulate that NF-κB activation could interfere with the metals' capacity to activate AhR (Fig. 3.16), resulting in suppression of the *Cyp1a1* gene. This postulation is supported by previous observations that PMA suppresses *CYP1A1* gene expression through decreasing nuclear AhR levels and hence the AhR-XRE binding (Moore et al., 1993; Guo et al., 2001).

To examine the role of the AP-1 signaling pathway in the modulation of the *Cyp1a1* gene by metals, we investigated whether the inhibition of AP-1 upstream signaling pathway activators such as JNK, ERK, and p38 MAPK modulates *Cyp1a1* gene expression by metals. The JNK, ERK, and the p38 are ser/thr protein kinases that comprise the family of MAPKs. MAPKs act as intracellular signaling mediators and hence controlling gene expression in the nucleus in response to changes in the cellular environment through

phosphorylation of transcription factors (Tan et al., 2004). Mechanistically, MAPKs are activated upon phosphorylation by a MAPK kinase (MAPKK), which in turn is activated when phosphorylated by MAPKK kinase (Raman et al., 2007).

Our results clearly suggest that activation of MAPKs signaling pathways may be required for metals to modulate the expression of the *Cyp1a1* gene in a metal- and MAPK-dependent manner. We showed here that JNK could be a major contributor to *Cyp1a1* modulation by metals whereas ERK negatively regulates the metal-mediated effects. For example, the inhibition of the JNK signaling pathway completely abolished metal-mediated induction of Cyp1a1 mRNA, but potentiated their inhibitory effects on Cyp1a1 induction at the activity level (Fig. 3.37). In contrast, ERK inhibition potentiated Cyp1a1 mRNA induction by metals, whereas completely reversed the inhibitory effect at the activity levels (Fig. 3.38). p38 MAPK inhibition, on the other hand, suppressed the metal-mediated induction of Cyp1a1 mRNA, but did not significantly alter the Cyp1a1 activity (Fig. 8). Based on these results, it is possible that metals activate the MAPKs signaling pathway, leading to phosphorylation of several transcription factors that participate in the *Cyp1a1* regulation. This postulation is supported by the observations that MAPK activation potentiates the activity of the AhR-XRE complex and regulates the activities of several transcriptional coactivators and/or corepressors (Tan et al., 2004). In this context, it has been reported previously that Hg^{2+}, Pb^{2+}, and Cu^{2+} are able to induce MAPKs activity and subsequently AP-1 activity (Mattie and Freedman, 2004; Walczak-Drzewiecka et al., 2005; Choi et al., 2006).

In conclusion, we showed here that activation of the NF-κB signaling pathway may negatively regulates the expression of the *Cyp1a1* gene by metals. Furthermore, activation of AP-1 and MAPK signaling pathways could play a critical role in the induction of *Cyp1a1* gene expression by all the metals tested. These differential effects of NF-κB and AP-1 on the modulation of the *Cyp1a1* gene by metals indicate that a complex regulatory mechanism exists, in which an imbalance in the regulation of cellular signaling pathways would determine the capacity of metals to modulate the expression of the *Cyp1a1* gene.

4.1.5. NF-κB and AP-1 Signaling Pathways Modulate the Expression of the *Nqo1* Gene by Hg^{2+}, Pb^{2+}, and Cu^{2+}

To further explore the role of redox-sensitive transcription factors, NF-κB and AP-1, in the modulation of phase II enzymes by metals, we investigated the effect of NF-κB and AP-1 modulators on the expression of the *Nqo1* gene by Hg^{2+}, Pb^{2+}, and Cu^{2+}. Since a similar pattern of modulation of *Nqo1* and *Gsta1* gene expression by metals was observed, the *Nqo1* gene was utilized in the current study as a useful indicator of overall modulation of phase II enzymes regulation by metals.

Initially, we examined whether or not GSH depletion would potentiate metal-mediated effects on *Nqo1* gene expression. Our results showed that cellular GSH depletion potentiated the induction of Nqo1 mRNA and activity levels by the three metals (Fig. 3.40). These results suggest that metal-mediated induction of the *Nqo1* gene is regulated through oxidative stress-mediated mechanisms.

Several studies reported the presence of potential binding sites for NF-κB and AP-1 within the ARE sequence that are responsible for the basal expression of NQO1. In addition, a mutation of the AP-1 binding site resulted in the loss of basal and inducible expression of NQO1 (Li and Jaiswal, 1992a; Li and Jaiswal, 1992b). In this regard, the ability of all three metals to induce the activation of NF-κB and AP-1 DNA binding activities in Hepa 1c1c7 cells (Fig. 3.34) prompted us to examine the role of these transcription factors in the modulation of Nqo1 by metals.

The possible involvement of NF-κB was assessed by determining the effect of the NF-κB activator and inhibitor, PMA and PDTC, respectively. In contrast to what is known about the role of NF-κB in the regulation of *NQO1*, we demonstrated that activation of NF-κB signaling pathways may negatively regulates the induction of the *Nqo1* gene in response to metals in Hepa 1c1c7 cells. PMA inhibited the metal-mediated induction of Nqo1 at the mRNA and enzyme activity levels (Fig. 3.35). In contrast, PDTC further increased the basal and Pb^{2+}-mediated induction of Nqo1 mRNA and activity levels, but did not alter other metal-mediated effects (Fig. 3.36). The negative regulatory effect of NF-κB could be attributed to the inhibitory interaction between the AhR and NF-κB signaling pathways (Tian et al., 1999; Kim et al., 2000; Tian et al., 2002).

Although the mechanism of interaction between NF-κB and the AhR is still undetermined, several hypotheses have been proposed (Tian et al., 1999; Ke et al., 2001; Tian et al., 2002). Several studies suggested that this cross-talk is primarily a cytoplasmic process, in that the inactivated AhR and NF-κB in the cytoplasm are kept away by being

sequestered by their inhibitory proteins, HSP90 and IκB, respectively. However once activated by TCDD and TNF-α, respectively, the AhR and NF-κB would then interact (Tian et al., 1999). In addition, transient transfection of Hepa 1c1c7 cells with the AhR did not alter IκB levels, suggesting that the repressive effects are not mediated through the induction of IκB (Tian et al., 1999). Other studies showed that NF-κB and the AhR could compete for transcriptional coactivators and corepressor proteins, suggesting that a nuclear cross-talk may carried out (Ke et al., 2001).

To characterize the role of AP-1, we investigated whether or not the inhibition of AP-1 upstream signaling pathway activators, JNK, ERK, and p38 MAPK, would modulate the *Nqo1* gene expression by metals. Our studies showed that the JNK signaling pathway upregulates the metal-mediated induction of *Nqo1* (Fig. 3.37). In contrast, activation of the ERK signaling pathway negatively regulates the expression of Nqo1 by metals (Fig. 3.38). On the other hand, inhibition of the p38 MAPK signaling pathway suppressed Hg^{2+}- while potentiating Pb^{2+}- and Cu^{2+}-mediated effects at the mRNA levels (Fig. 3.39). Based on these results, it is possible that metals differentially activate the MAPKs signaling pathway leading to phosphorylation of several transcription factors that participate in AhR and ARE activation and subsequent *Nqo1* induction (Tan et al., 2004). In this context, it has been reported previously that Hg^{2+}, Pb^{2+}, and Cu^{2+} are able to induce MAPKs activities and subsequently AP-1 activity (Mattie and Freedman, 2004; Walczak-Drzewiecka et al., 2005; Choi et al., 2006).

A cross-talk between the AhR and AP-1 signaling pathways has been previously reported (Hoffer et al., 1996; Suh et al., 2002). TCDD inhibited LPS-induced DNA binding and transcriptional activity of AP-1 in murine lymphoma WT CH12.LX, but not in AhR-deficient BCL-1 cells (Suh et al., 2002). In addition, treatment of Hepa 1c1c7 cells with TCDD caused an increase in c-fos and c-jun mRNA levels, which was associated with an increase in the DNA-binding activity of AP-1 (Hoffer et al., 1996), suggesting that AP-1 activation requires a functional AhR-XRE complex.

We conclude that the NF-κB signaling pathway negatively regulates the expression of the *Nqo1* gene by metals. We showed here that activation of NF-κB suppressed, whereas inhibition of the NF-κB signaling pathway further potentiated the metal-mediated induction of *Nqo1* gene expression. Furthermore, activation of AP-1 and MAPK signaling pathways are critical for the induction of *Nqo1* gene expression by all metals.

4.2. GENERAL CONCLUSION

Long-term human exposure to metals is a common environmental problem with persistent biological consequences, including effects on the xenobiotic metabolizing systems. Previous studies have suggested that environmental co-contamination of metals and PAHs could enhance or reduce the carcinogenicity of PAHs by modifying the expression of AhR-regulated genes (Maier et al., 2000; Kann et al., 2005). Yet, the effects and the mechanisms invloved remain unknown.

In the current study, we have demonstrated that the metals, particularly Hg^{2+}, Pb^{2+}, and Cu^{2+} differentially modulate the constitutive and inducible expression of *Cyp1a1*, *Nqo1*, and *Gsta1* genes. Our data clearly showed that metals may decrease the carcinogenicity and muatgenicity of AhR ligands by decreasing the induction of *Cyp1a1* while enhancing the induction of *Nqo1* and *Gsta1* genes. Particularly, co-administration of AhR ligands with Hg^{2+} or Pb^{2+} significantly potentiated Nqo1 and Gsta1 at the mRNA and activity levels. However, both metals inhibited the induction of Cyp1a1 by AhR ligands, while increasing its mRNA expression. Cu^{2+}, on the other hand, inhibits the induction of Cyp1a1 by AhR ligands at the activity and mRNA levels, and Nqo1 at the activity and mRNA levels. Furthermore, all metals increased the expression of HO-1 which coincided with the decrease in phase I and the increase in phase II activities.

The current study provided the first evidence that metals modulate *Cyp1a1* gene expression at the transcriptional and post-transcriptional levels through an AhR-dependent pathway. The transcription inhibitor, Act-D, completely blocked the metal-mediated induction of Cyp1a1 indicating a requirement of *de novo* RNA synthesis, whereas the protein synthesis inhibitor, CHX, superinduced the metal-mediated effect. Furthermore, the Cyp1a1 mRNA and protein decay experiments showed that the three metals did not significantly affect the half-life of mRNA; however, they significantly decreased the degradation rate of its protein, implying a post-translational regulation of the Cyp1a1 by the metals.

Several pieces of evidence support the AhR-dependent induction of Cyp1a1 by metals. First, lack of Cyp1a1 induction in response to metals in AhR deficient C12 cells; second, inhibition of AhR degradation by the 26S proteasome inhibitor, MG-132, increased the metal-mediated effects; and third, the ability of all metals to directly bind to and activate the AhR-XRE pathways strongly support an AhR-dependent mechanism.

The three metals induced the expression of *Nqo1* and *Gsta1* genes at the transcriptional level through both AhR-XRE- and Nrf2-ARE-dependent mechanisms. Blocking the RNA synthesis by Act-D significantly blocked the induction of these genes by metals. In addition, the protein synthesis inhibitor CHX significantly inhibited the metal-mediated induction of Nqo1 and Gsta1 at mRNA level. This coincided with a decrease in Nrf2 protein expression, implying the requirement of Nrf2 protein synthesis for the induction of these genes. Furthermore, inhibition of Nrf2 protein degradation by the 26S proteasome inhibitor, MG-132, significantly prevented the CHX-mediated inhibition of Nqo1 and Gsta1 mRNAs, which coincided with an increase in the expression of Nrf2, confirming that a transcriptional mechanism is involved.

The current study provides strong evidence for the involvement of NF-κB and AP-1 signaling pathways in the modulation of AhR-regulated genes by metals. Our results demonstrated that the NF-κB signaling pathway negatively regulates the expression of *Cyp1a1* and *Nqo1* genes by metals. The NF-κB activator abolished, whereas the NF-κB inhibitor further potentiated, the metal-mediated effects on *Cyp1a1* and *Nqo1* genes. On the other hand, activation of AP-1 and MAPK signaling pathways are critical for the

induction of *Cyp1a1* and *Nqo1* genes by metals. We showed here that JNK is the major contributor to the metal-mediated induction of *Cyp1a1* and *Nqo1*, whereas ERK negatively regulates the metal-mediated effects. These results strongly suggest a cross-talk between the AhR and redox-sensitive transcription factors. The differential effects of NF-κB and AP-1 on the modulation of AhR-regulated genes by metals indicate that a complex regulatory mechanism exists, in which an imbalance in the regulation of cellular signaling pathways would determine the capacity of metals to modulate the expression of AhR-regulated genes.

4.3. FUTURE DIRECTION AND STUDIES

The results obtained from the current research raise several questions that need to addressed and answered in order to increase our understanding about the potential interactions between metals and AhR ligands. Therefore, further studies need to be conducted to:

1. determine the effect of coexposure of metals and AhR ligands on the AhR-regulated genes *in vivo*.
2. investigate whether the modulation patterns of the AhR-regulated genes by metals are tissue-specific. Therefore, expression of these genes in different organs and tissues should examined at the mRNA, protein, and activity levels.
3. examine the role of coregulatory proteins such as coactivator and corepressor proteins in the modulation of AhR-regulated genes by metals.

4. identify the genes that are induced by metals and mediate the cross-talk between the AhR and redox-sensitive transcription factors using global microarray analysis.

CHAPTER 5- REFERENCES

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